THE EFFECT OF ALCOHOL TOXICOLOGY IN THE FORM OF DIFFERENT ALCOHOL DRINKING PATTERNS ON A BIOMARKER OF CARDIOVASCULAR DISEASE RISK

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Abbreviations

Abbreviation	Meaning
ABV	Alcohol by Volume
%ABV	Percentage Alcohol by Volume
ADH	Alcohol Dehydrogenase
ADMA	Asymmetrical Dimethylarginine
ADI	Alcohol Dependent Individual
AdoCbl	Adenosylcobalamin
ALT	Alanine Aminotransferase
AM	Alcohol Manufacturer
APS	Alcohol Problems Service
APRI	Platelet Ratio Index
ApoB	Apolipoprotein B
ApoJ	Plasma sialic acid index of apolioprotein
AST	Aspirate Aminotransferase
Au	Gold
AUDIT-C	Alcohol Use Disorder Identification Test – Consumption
AUDIT	Alcohol Use Disorder Identification Test
AUDs	Alcohol Use Disorders
℃	Degree Celsius
β	Beta
β-Hex	Beta-hexosaminidase
BAC	Blood Alcohol Concentration
BHF	British Heart Foundation
BHMT	Betaine-Homocysteine-Methyltransferase
BUPA	British United Provident Association
CC	Cytosine-Cytosine
CT	Cytosine-Thymine
C677T	Cytosine to Thymine transition at nucleotide 677
CAD	Coronary Artery Disease
$CaCl_2$	Calcium Chloride
CAGE	Cut-down, Annoyed, Guilt, Eye-opener
Cbl	Cobalamin

Abbreviation	Meaning
CBS	Cystathionine β-synthase
cDNA	Copied Deoxyribonucleic acid
CDG	Carbohydrate Deficient Glycoprotein
CDT	Carbohydrate Deficient Transferrin
CE	Capillary Electrophoresis
CE-LIF	Capillary Electrophoresis-Laser Induced Fluorescence
CHD	Coronary Heart Disease
CH_3	Methyl group
CONSORT	Consolidated Standards of Reporting Trials
CV	Coefficient of Variation
CVD	Cardiovascular Disease
CYS	Cystathionase
4A1	Cytochrome P450 Enzyme
2E1	Cytochrome P450 Enzyme
Da	Dalton
DALYS	Disability Adjusted Life Years
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithlotheritol
DSM	Diagnostic and Statistical Manual of Mental Disorders
DVT	Deep Vein Thrombosis
ED	Electrochemical Detection
EDTA	Ethylenediaminetetraacetic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immuno-Sorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
EPPF	Edinburgh Protein Production Facility
EtG	Ethyl Glucuronide
EtS	Ethyl Sulphate
FA	Fatty Acid
FAEEs	Fatty Acid Ethy Esters
FDA	Food and Drug Administration
$FeCl_3$	Ferric Chloride

Abbreviation	Meaning
FeNTA	Ferric Nitrilotriacetic Acid
FMD	Flow Mediated Dilation
FIPA	Fluorescence Polarized Immunoassay
FY1	First Year Doctor
γ	Gamma
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
g	Grams
g	Relative Centrifugal Force
g/l	Grams per litre
GBD	Global Burden of Disease
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass Spectrometry
GC-ID-MS	Gas Chromatography – Isotopic Dilution – Mass Spectrometry
GDP	Gross Domestic Product
GI	Gastrointestinal Tract
GGT	Gamma-Glutamyltransferase
GP	General Practioner
GSCbl	Glutathionylcobalamin
HAA	Haemoglobin Associated Acetaludehyde
HI	Healthy Individual
HIV	Human Immunodeficiency Virus
HCl	Hydrochloric Acid
Hcy-LDL	Homocystamide-Low Density Adduct
HcyTa	Homocysteine Thiolactone
HED	Heavy Episodic Drinking
HDL	High Density Lipoprotein
HOPE-2	Healthy Outcomes Prevention Evaluation 2
HoloTc	Holotranscoloabalmin
HPLC	High Pressure Liquid Chromatography
HPLC-ED	High Pressure Liquid Chromatography with Electrochemical Detection
HPLC-FD	High Pressure Liquid Chromatography with Fluorescence Detection

Abbreviation	Meaning
HTOL/5-HIAA	5-hydroxytryptophol/5-hydroxyindole acetic acid ratio
ICL	Immuno-Chemiluminscence
ICD	International Classification of Diseases
IDL	Intermediate Density Lipoprotein
IEC	Ion-Exchange Chromatography
IFCC	International Federation of Clinical Chemistry
IFCC-WG-CDT	International Federation of Clinical Chemistry Working Group on Carbohydrate Deficient Transferrin
IFCCLM	International Federation of Clinical Chemistry and Laboratory Medicine
ISD	Information Services Division
JACC	Japan Collaborative Cohort
kDa	Kilo Dalton
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectroscopy
LC-MRM/MS	Liquid Chromatography-Multiple Reaction Monitoring-Mass Spectrometry
LDL	Low Density Lipoprotein
L-FABP	Liver Cystolic Fatty Acid Binding Protein
LREC	Lothian Research Ethics Committee
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
MAO-B	Monoamine Oxidase - Type B
MAT	Methionine Adenosyltransferase
MAST	Michigan Alcohol Screening Test
mAU	Milli Absorbance Unit
MEOS	Microsomal Ethanol Oxidizing System
MeCbl	Methylcobalamin
MS	Mass Spectroscopy
MS	Methionine Synthase
MTRR	Methionine Synthase Reductase
µg	Micrograms
µg/l	Micrograms per litre
µl	Micro litres

Abbreviation	Meaning
µmol/l	Micromoles per litre
ml	Millilitres
mMol	Millimoles
mg	Milligrams
M	Molar
mm	Millimetre
Mol/l	Moles per litre
MCV	Mean Corpuscular Volume
mBrB	Monobromobimane
MTHF	Methylenetetrahydrofolate
MTHFR	Methylenetetrahydrofolate Reductase
MI	Myocardial Infarction
N	Number of participants
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced form of Nicotinamide Adenine Dinucleotide.
nA	Nano Amps
ng/l	Nano grams per litre
nm	Nano metre
$NaBH_4$	Sodium Borohydride
NaOH	Sodium Hydroxide
NET	Normal drinker, Eye-opener, Tolerance
NF-KB	Nuclear Factor-KappaB
NHANES	National Health and Nutrition Examination Survey
NHS	National Health Service
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NICE	National Institute of Clinical Excellence
NO	Nitric Oxide
N_2O	Nitrous oxide
NORVIT	Norwegian Vitamin Trial
NTA	Nitrilotriacetic
OPA	O-phthaldialdehyde
ONS	Office of National Statistics
OPS	O-phthaldialdehyde

Abbreviation	Meaning
PPP	Purchasing Power Parity
PAI	Plasminogen-activator inhibitor
PABA	p-aminobenzoic acid
PCR	Polymerase Chain Reaction
PDA	Personal Digital Assistant
PEth	Phosphatidyl Ethanol
pg	Picograms
%	Percentage
%CV	Percentage Coefficient of Variation
pH	$-\log_{10}[H^+]$
PhD	Doctor of Philosophy
QMU	Queen Margaret University
R	Correlation Coefficient
R^2	Coefficient of Determination
RCT	Randomised Control Trial
RDI	Recommended Daily Intake
RIE	Royal Infirmary of Edinburgh
RLU	Relative Light Units
rpm	Revolutions Per Minute
SAH	S-adenosylhomocysteine
SAHase	S-adenosyl-L-homocysteine hydrolase
SAHH	S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethionine
SD	Standard Deviation
SIJ	Sialic acid index of plasma apolipoprotein J
SMAST	Short Michigan Alcohol Screening Test
SNP	Single Nucleotide Polymorphism
SOS	Sodium Octyl Sulphate
STI	Sexually Transmitted Infection
t-pa	Tissue-plasminogen activator
TC	Transcobalamin
TT	Thymine-Thymine
T-ACE	Take (number of drinks), Annoyed, Cut-down, Eye-opener

Abbreviation	Meaning
TC/Cbl	Transcobalamin/Cobalamin Complex
THF	Tetrahydrofolate
TBE	Tris-Borate Ethylenediaminetetraacetic Acid
TSA	Total sialic acid
TWEAK	Tolerance, Worried, Eye-opener, Amnesia, Kut-down
UPSTF	US Preventive Services Task Force
USA	United States of America
USD	United States Dollar
UV	Ultra Violet
UK	United Kingdom
V	Volts
VLDL	Very Low Density Lipoprotein
WD	WinDiets Dietary Software
WG-CDT	Working Group on Carbohydrate Deficient Transferrrin
WHO	World Health Organisation

Declaration

I declare that the work contained within this thesis is original. I have solely been responsible for the organisation of the study herein, as well as all aspects of data collection and the analysis of results, unless otherwise stated.

Julie M. Murdoch

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Abstract

Introduction

The amino acid homocysteine has been identified as a risk factor for cardiovascular disease (CVD), as elevated levels induce atherosclerosis, through a direct effect on arterial tissue. In alcohol dependent individuals an association between plasma homocysteine levels and alcohol consumption has been found (Bleich et al. 2000d). However this link has not been explored in individuals who have a range of non-dependent alcohol drinking patterns. This fact has informed the design of the present work.

Material and Methods

A convenience sample of abstainers (N=7), non-dependent drinkers (N=28) and alcohol dependent individuals (N=18) was recruited. Alcohol consumption was recorded using questionnaires and diaries. All study participants' biological samples were analysed for the following biomarkers: plasma homocysteine (HPLC); serum folate and vitamin B_{12} (competitive immunoassay); serum Carbohydrate Deficient Transferrin (N-Latex immunoassay); urinary creatinine (colorimetric assay) and the methylenetetrahydrofolate ($MTHFR_{C677T}$) polymorphism (Real-Time Polymerase Chain Reaction (PCR)).

Results

There was no association between alcohol consumption during drinking days and plasma homocysteine levels in non-dependent drinkers. However when this group was categorised according to pattern of consumption, plasma homocysteine levels were found to be lower in abstainers (median 5.60 µmol/l), but higher in sessional drinkers (median 7.15 µmol/l) and alcohol-dependent individuals (median 7.89 µmol/). The mutant $MTHFR_{(C677T)}$ polymorphism when present was associated with an increase in plasma homocysteine levels, which correlated with alcohol consumption (R=0.975). CDT levels were found to be elevated in both sessional and alcohol-dependent individuals at baseline (median 2.68% and 5.95% respectively) compared with abstainers (2.16%). Additionally there was a linear relationship between the number of drinking days in a week and a positive CDT result, in a sample of sessional drinkers (R=0.98).

Discussion

The work undertaken has shown that sessional drinking and alcohol dependence does increase homocysteine levels in comparison to abstainers. This may have important implications in relation to CVD risk. Additionally new evidence of the utility of CDT as a biomarker of alcohol consumption within a sample of sessional drinkers, has been identified.

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

The misuse of alcohol remains a major public health issue within the UK, being linked to a wide range of medical conditions including increased risk of cardiovascular disease (CVD) (Agarwal 2002; Lakshman et al. 2009). A review by (Anderson et al. 2009), further highlights the effect of alcohol as a casual factor on the following: intentional, unintentional injuries and harm to people other than the drinker (Rehm et al. 2004), reduced job performance (Manigone et al. 1999), absenteeism (Roche et al. 2008), family deprivation (Gururaj et al. 2006), interpersonal violence (Gil-González et al. 2006), suicide (Cargiulo 2007), murder (Rehm et al. 2006), crime (Richardson and Budd 2003) and fatalities caused by driving while under the influence of alcohol (drink driving) (Cherpitel et al. 2003). Furthermore alcohol has also been shown to be a contributing factor to undertaking risky sexual behaviour (Kalichman et al. 2007), sexually transmitted infections (STIs) (Cook and Clark 2005) and Human Immunodeficiency Virus (HIV) infection (Fisher et al. 2007). The consumption of alcohol, especially in specific drinking patterns, requires further investigation as to its impact on health. Within the literature there are a number of studies which identify the costs of alcohol misuse, which are useful in identifying the greatest economic costs caused by alcohol misuse and the specific problems generated by alcohol misuse and in which geographical regions (Mohapatra et al. 2010; Rehm et al. 2009).

Alcohol is a risk factor for global burden of disease (GBD) and is ranked fifth, behind tobacco (Ezzati et al. 2004). New data has been published in 2010, by a key group of UK alcohol and drug researchers and have categorised alcohol as the number one drug, for causing harm, above the illegal drugs such as heroin, crack cocaine and metamfetamine (Nutt et al. 2010). European regions have the highest proportion of alcohol attributable deaths with more than one in every ten deaths seen in European men. Within Europe, the highest proportion was for the countries of the former Soviet Union (Rehm et al. 2009). Scotland is ranked eighth in the world for the highest pure alcohol consumption (The Scottish Government 2009b). The per capita pure alcohol consumption in Scotland is 11.8 litres, which is higher in comparison to England and Wales which is 10.3 litres (The Scottish Government 2009b). There is a close link between a country's total alcohol per head consumption and the prevalence of alcohol-related harm and alcohol dependence;

when alcohol consumption increases so does alcohol-related harm and the proportion of people with alcohol dependence. Furthermore there appears to be an association between the prevalence of alcohol dependence and alcohol related harm. The death of 1 in 20 Scots has been linked to alcohol related causes (The Scottish Government 2009a).

The effect of alcohol consumption on health is estimated at 3.8% of all global deaths and 4.6% of global disability-adjusted life years (DALYs) are directly attributable to alcohol (Rehm et al. 2009). The costs associated with alcohol amount to more than 1% of the gross national product in high-income and middle-income countries and the costs of social harm constitute a major proportion in additional health costs (Rehm et al. 2009). Alcohol misuse costs the UK economy £6.4 billion, through alcohol related sickness, absence from work and death (Ward et al. 2010). Table 1.1 illustrates the cost burden of alcohol misuse in Scotland, compared with other countries around the world, and all are classified as high income countries.

It is clear from table 1.1 that Scotland incurs similar healthcare costs as a percentage of total social costs, to that of the USA, however these costs are for populations which are 54 times smaller than the total USA population. The high level of alcohol induced costs to law enforcement in Scotland, are shown to be higher than the largest sample country, the USA. Both these statistics are worrying and highlight the detrimental problem alcohol misuse is having on societal costs within Scotland. Further highlighted in table 1.1, Scotland and the USA have the highest total alcohol costs as a percentage of the gross domestic product (GDP) (purchasing power parity (PPP)), in comparison to other high income countries, including Australia, Canada and France. Italy has the lowest alcohol costs as a percentage of their GDP.

Table 1.1: Overview of social costs attributable to alcohol in 7 developed countries (Mohapatra et al. 2010)

Indicators	Australia	Canada	France	Italy	UK (Scotland)	USA
Reference	(Collins and Lapsley 2002)	(Rehm et al. 2006)	(Fenoglio et al. 2003)	(Collicelli 1996)	(Varney and Guest 2002)	(US Department of Health and Human Services 2000)
Population in study year (millions)	18.8	31.3	58.7	56.8	5.1	278.4
GDP (PPP) IN 2006 million USD	674,600	1,181,000	1,902,000	1,756,000	165,808	13,060,000
Total social costs of alcohol	5,227.6	13,957.9	25,347.4	11,573.5	3555.7	216,154.5
Healthcare costs as % of total social cost	4.1	22.7	16.0	19.9	8.9	13.3
Law enforcement costs as % of total social costs	15.5	21.1	0.3	1.3	25.0	3.4
TOTAL alcohol costs as % GDP (PPP)	0.77	1.18	1.33	0.66	1.36	1.66
HEALTHCARE alcohol costs as % GDP (PP)	0.03	0.27	0.21	0.13	0.12	0.22
LAW alcohol costs as % GDP (PPP)	0.12	0.25	0.00	0.01	0.34	0.06

A more recent report by the Scottish Government has shown the estimated societal costs of alcohol misuse in Scotland from 2007 is approximately £3.55 billion per year, which equates to approximately £500 for every adult in Scotland (The Scottish Government 2010b). The prevalence of harmful and hazardous alcohol use (defined in table 1.3), in Scotland, as stated in the 2003 Scottish Health Survey was 27.9% (Drummond et al. 2009). The prevalence of alcohol dependence in Scotland was 4.9% in adults over the age of 16 years (Drummond et al. 2009). These percentages indicate that approximately 1,172,200 people were drinking either harmfully or hazardously and approximately 206,000 were dependent on alcohol (Drummond et al. 2009). Table 1.2 illustrates the estimated costs of alcohol misuse within major social resources, up to 2007.

Table 1.2: Estimated annual societal costs of alcohol misuse in Scotland for 2007 (The Scottish Government 2010b)

Resource	**Range (£million)**	**Mid-point (£million)**
Health Service	143.6-392.8	267.8
Social Care	114.2-346.8	230.5
Crime	462.5-991.7	727.1
Productive Capacity of The Scottish Economy	725.2-1006.1	865.7
Wider Social Costs	1031.1-1898.0	1464.6
Total	**2476.6-4635.4**	**3555.7**

Table 1.2 clearly shows the effect of alcohol misuse is increasing the burden on NHS Scotland and the other core social resources. An approximate total cost of alcohol misuse on the UK NHS system has been estimated at £3 billion, by the leading alcohol charity, Alcohol Concern (Ward et al. 2010). This figure encompasses the approximate cost of treating alcohol dependency, which is estimated to cost the UK NHS £217 million (Drummond 2005). This figure refers to the treating of just 6% of alcohol dependent patients. In comparison it costs the UK NHS £436 million (Department of Health 2009) to treat 58% of drug dependent patients. If 50% of all alcohol dependent patients were treated for their dependency problem; this would cost the NHS billions of pounds. Furthermore it is clear from the

figures generated from the report by Alcohol Concern that alcohol dependency is more expensive to treat in comparison to drug dependency (Ward et al. 2010).

Recent figures have shown that within the last ten years, UK hospital admissions due to alcohol attributed conditions have doubled (Song 2009; The NHS Information Centre 2008) This statistic highlights the detrimental effects acute and chronic alcohol consumption have on the health of the Scottish population. In 2007/2008 there were 42,430 alcohol related discharges from general hospitals in Scotland, this is equal to 777 per 100,000 of the population (The Scottish Government 2010a). Of the 42,430 total, 28,586 patients were discharged with an alcohol-related diagnosis for their admission to hospital, which included 6817 discharges for alcoholic liver disease (NHS Scotland 2009).

The impact of alcohol misuse, does not only effect the NHS, but also has a cost burden on the other societal resources, including the criminal justice system and social services (Department for Work and Pensions 2010). The effect of alcohol misuse on the family unit can cause a great impact, for example: increased family tension, increased levels of quarrelling and violence; destabilising relationships, family members becoming anxious, depressed, socially withdrawn or developing their own alcohol misuse problems; furthermore children can develop behavioural problems and underperform at school (Department for Work and Pensions 2010). Alcohol misuse has also be linked to increasing rates of marriage separation and divorce (Department for Work and Pensions 2010). The burden of alcohol misuse on the family unit can also overflow into the affected individual's workplace, whereby deterioration in performance, conflict with colleagues, an increase in workplace accidents and poor attendance, could lead to dismissal and lack of employability (Department for Work and Pensions 2010). There is also potential for these outcomes to affect an individual, who is not misusing alcohol, but lives with a family member who has an alcohol problem. Another major burden of alcohol misuse is on the criminal justice system, where heavy drinking is associated with crimes including: petty theft, driving offences, fraud, sexual offences and violent crime (Department for Work and Pensions 2010). A report by the Scottish Government has shown the increasing costs of alcohol misuse on the criminal justice and emergency services and social work services from, 2001 to 2007, where there has

been a 69% and 50% increase in costs respectively since 2001 (The Scottish Government 2010b).

In an effort to combat excessive alcohol consumption and alcohol misuse in Scotland, the Scottish Government has prepared a bill to tackle the effect alcohol misuse has on society across Scotland (The Scottish Government 2010a). The "Alcohol Bill" has several key proposals which include: minimum pricing of alcohol; bans on irresponsible marketing and promotions in bars and clubs; ensuring the availability of small measures of wine in licensed premises; increasing the purchase age of alcohol off sales to 21 years and potentially charging alcohol retailers a social responsibility fee (The Scottish Government 2010a). The Alcohol Bill has been presented to the Scottish Parliament in early 2010 and as of October 2010 the bill is at the second stage of development, with a view of being made law in 2011. Furthermore the National Institute for Health and Clinical Excellence (NICE) has issued guidelines which aim to reduce alcohol related harm (National Institute for Health and Clinical Excellence (NICE) 2010). The guidelines, published on the 2nd June 2010 state:

- Alcohol should be made less affordable by introducing a minimum price per unit. Prices should also be regularly reviewed.
- Alcohol should be made less easy to buy, e.g. reducing the volume of imports, alcohol outlets within a given area and the hours in which alcohol can be purchased.
- Applications for new licences to sell alcohol should be based on the number of outlets in a given area, taking into consideration the potential impacts on crime, disorder and alcohol related illnesses and deaths.
- "Protection of the public's health" should be added to the current licensing objectives, which are already the case in Scotland. This would mean that premises would have a legal obligation to consider the health of their customers when selling them alcohol.
- The current advertising regulations should be strengthened to minimise children and young people's exposure to alcohol products. A complete ban on alcohol advertising should also be considered to protect these high-risk groups, as in the case with tobacco products.

The guidelines issued by NICE and the Scottish Governments Alcohol Bill, highlight the efforts to reduce alcohol related harm.

In 2004 most of the global alcohol-attributable burden of disease occurred in people age 15-29 years (33.6% of all alcohol-attributable DALYs) followed by those aged 20-44 years (31.3%) and 45-59 years (22.0%) (Rehm et al. 2009). In all these age groups, alcohol consumption was responsible for more than 10% of all burden of disease in men and for 2-3% in women (Rehm et al. 2009). When compared with other traditional risk factors including tobacco, cholesterol or hypertension the age profile of alcohol-attributable disease burden is shifted towards younger populations (Rehm et al. 2009).

Figures relating to 2005 suggests, 88% of Scottish men and women consume alcohol (Catto 2008). More recent figures show that in Scotland it has been estimated that 40% of men and 33% of women consume more than the recommended daily alcohol drinking limits (guidelines explained in section 1.2) (British Heart Foundation Health Promotion Research Group 2008). In relation to specific age groups, The Alcohol Statistics Scotland 2009 report stated than 60% of 16-24 year old males and females exceed the Governments drinking guidelines at least once per week. This statistic from 2009 shows a continuing trend from 2004, where alcohol attributable death is shifting from an older population to a younger population and highlights the need to investigate alcohol drinking patterns in younger age groups.

A review published by Coghill et al. (2009) examined the levels of per capita alcohol consumption and alcohol-related mortality in the UK over the past 25 years and found that there was a significant correlation (which has increased from 1991 to 2006) between per capita alcohol consumption and alcohol-related mortality per 100,000 of the population (Coghill et al. 2009). The report also showed that the correlation between alcohol consumption-related morbidity was highest in Scotland, and the full effects of consumption became apparent, approximately one year later (Coghill et al. 2009).

Exceeding the recommended alcohol drinking guidelines increases the risk of coronary heart disease (CHD) and research has shown that 40% of young men and 34% of young women exceed these guidelines at least once per week in Scotland (British Heart Foundation Health Promotion Research Group 2008), putting them at risk of developing CHD or another associated CVD. A report by Rehm et al. (2009)

has highlighted that two different types of alcohol consumption affect health: average volume of alcohol consumption and patterns of drinking, especially episodes involving heavy drinking (Rehm et al. 2003; Rehm et al. 2004). Drinking patterns which are associated with heavy consumption (e.g. sessional drinking or heavy episodic drinking) are more common in countries with a lower GDP in comparison to countries with a higher GDP. This can be illustrated through the different life expectancies between eastern and western Europe (Anderson, Chisholm and Fuhr 2009; Zatonski 2008). Scotland's alcohol consumption behaviour, contradicts this trend as Scotland is classified by the World Health Organisation (WHO) as a high-income country, but has a high rate of alcohol consumption, especially in terms of sessional drinking (The Scottish Government 2009b).

A study conducted by Information Services Division (ISD) Scotland found that 1493 deaths from heart disease could have been prevented by lower levels of alcohol consumption (The Scottish Government 2009a). Scotland has the highest rates of CHD in the world and the second highest in the western world as reported in the Scottish Health Survey 2003 (Bromley et al. 2003). Statistics generated from research conducted more recently by the British Heart Foundation (BHF) highlighted that there were 18,000 CVD deaths in Scotland in 2008/2009 (British Heart Foundation Health Promotion Research Group 2008). The report by the British Heart Foundation also highlighted that within Scotland, 620,000 people have been diagnosed with heart and circulatory disease and there are approximately 12,000 individuals who suffer a myocardial infarction (MI) every year.

A known risk factor for CVD, has been identified as the amino acid homocysteine. Elevated levels of homocysteine induce atherosclerosis, through a direct effect on arterial tissue (McCully 2001). It has been suggested that there is an association between plasma homocysteine levels and alcohol consumption in alcohol dependent individuals (Bleich et al. 2000d). However this research has not been conducted in a population of healthy individuals who consume alcohol in a range of non-dependent patterns.

1.2 ALCOHOL CONSUMPTION GUIDELINES

Guidelines for the consumption of alcohol have been outlined by the UK Department of Health (UK Department of Health 1995) and act as a public health message. The consumption of alcohol above these recommended guidelines has been associated with detrimental health effects (Beaglehole and Bonita 2009). Different patterns of alcohol consumption can be described in terms of the UK unit of alcohol (8 g of ethanol).

1.2.1 Terminology and Definitions of Alcohol Drinking Patterns

A range of terminology in common usage is, used to describe the consumption of alcohol. A key report published by the British Medical Association (British Medical Association Board of Science 2008), has given specific definitions to alcohol terminology in common usage in conjunction with the World Health Organisation (WHO) International Classification of Diseases 10th revision (WHO ICD-10) (World Health Organisation 2007) report, and are described in table 1.3.

The terminology described in table 1.3 is commonly used by clinicians to diagnose patients who exhibit problems with alcohol dependence and have exhibited social and health problems, caused by excessive alcohol consumption. These terms should not be confused with alcohol drinking patterns which use specific numerical values to quantify the amount of alcohol being consumed.

Table 1.3: Terminologies commonly used to describe alcohol consumption (sourced from; British Medical Association Board of Science 2008; World Health Organisation 2007; World Health Organisation 2008; Drummond et al. 2009).

Alcohol Consumption Terminology	Definition
Alcohol abuse	A term in common usage, especially in the USA. Is not included within the ICD-10. The Diagnostic and Statistical Manual of Mental Disorder (DSM-IV, 4th Ed, 1994) defines psychoactive substance abuse as a maladaptive pattern of substance use leading to clinically significant impairment or distress, as manifested by one or more of the following occurring within a 12 month period: • Recurrent substance use resulting in a failure to fulfil major roles obligations at school, work or home. • Recurrent substance use in situations in which it is physically hazardous (e.g. driving a car) • Recurrent substance-related legal problems (e.g. arrests for being drunk and disorderly) • Continued substance use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the substance (e.g. arguments with spouse or physical fights).
Alcohol misuse	The use of alcohol for a purpose not consistent with legal or medical guidelines (World Health Organisation 2008).
Alcoholism	A term in common usage which is generally taken to refer to chronic continual drinking or periodic consumption of alcohol which characterized by impaired control over drinking, frequent episodes of intoxication, preoccupation with alcohol and the use of alcohol despite adverse consequences. Alcoholism is **not** included as a diagnostic term within the ICD-10.

Alcohol Consumption Terminology	Definition
Alcohol Use Disorders (AUDs)	AUDs are classified into three categories: hazardous alcohol use, harmful alcohol use and alcohol dependence. **Hazardous Alcohol Use**: A pattern of alcohol consumption that increases the risk of harmful consequences for the individual. In contrast to harmful use, hazardous drinking refers to patterns of use that are of public health significance despite the absence of any current disorder in the individual user. Hazardous drinking is **not** included as a diagnostic term in the ICD-10. For reference the current UK Government's responsible drinking guidelines are adult men should not exceed four units per day or 21 units per week and adult women should not exceed three units per day or 14 units per week (UK Department of Health 1995). **Harmful Alcohol Use**: A pattern of alcohol use that causes damage to physical and/or mental health and can include adverse social consequences. Harmful drinking is included as a diagnostic term within the ICD-10. **Alcohol Dependence Syndrome**: Classified by the ICD-10 as a cluster of behavioural, cognitive, and physiological phenomena that develop after repeated alcohol use and that typically include a strong desire to consume alcohol, difficulties in controlling its use, persisting in its use despite harmful consequences, a higher priority given to its use than to other activities and obligations, increased tolerance and sometimes a physical withdrawal state. AUDs are included in the World Health Organisation's International Classification of Mental and Behavioural Disorders (World Health Organisation 1992).

The use of the above terms to describe alcohol consumption does not allow for a quantitative measurement of the actual grams of alcohol consumed. The use of the phrase 'alcohol misuse' or 'alcohol abuse' does not quantitatively define the exact amount of alcohol consumed. The use of alcohol drinking pattern categories, which uses numerical values, such as grams of alcohol, within their definitions, allows for analysis of alcohol consumption to quantitatively determine if an individual is consuming alcohol in an unhealthy and potentially harmful pattern. By using set guidelines, such as the UK Department of Health's responsible daily drinking message of 16-24 g (female) and 24-32 g (male), allows the public to determine and monitor their consumption within a set range.

The review of the literature within this thesis will focus on the alcohol consumption guidelines which use exact grams of alcohol, recommended by the UK Department of Health, to determine if alcohol consumption is above or below the recommended daily limit. It is widely acknowledged in the literature that alcohol consumption definitions vary widely, however by using set definitions with numerical values this can reduce error, especially when comparing alcohol consumption to potential health risk by way of biological markers (e.g. plasma homocysteine).

Published literature within the field of alcohol research, often described alcohol consumption as, light, moderate or heavy, however these terms are not quantitative in terms of actually describing alcohol consumption in terms of units or grams of pure ethanol (Agarwal 2002; Agarwal and Srivastava 2001; Berger et al. 1999). The terms of light and moderate alcohol consumption have been widely used internationally and are unhelpful. Table 1.4 describes the currently accepted description of light, moderate, heavy and sessional drinking (Agarwal 2002).

Table 1.4: Comparison of UK units/day and grams of pure alcohol/day in light/moderate/heavy drinking

Descriptive words to describe alcohol consumption	Grams (g) of pure alcohol/day	UK Units*/day
Heavy	>80g	>10
Sessional	48-64g	>6-8
Moderate	30-80g	4-10
Light	<30 g	<4

* 1 unit = 8g of pure ethanol

It could be suggested that within the UK, moderate drinking can be defined as responsible drinking, whereby the Government has identified units (which can be converted to grams) of alcohol which are within healthy limits for both males and females (UK Department of Health 1995). However as the definitions of "light" and "moderate" drinking are not specific, but used within the literature frequently, without quantitative definition. The UK Department of Health guidelines for alcohol consumption, allows for the definition and quantification of drinking patterns such as responsible and sessional, as they provide known and specific alcohol quantities (units) within each definition. These pre-defined drinking patterns allows for alcohol consumption to be equated to potential health risks, including CVD.

1.2.2 Responsible Drinking

Within the UK, the Department of Health has put in place guidelines for the responsible consumption of alcohol (UK Department of Health 1995). This report was in response to evidence which was published in the literature which suggested that moderate alcohol consumption could give protection from CHD (Agarwal 2002; Agarwal and Srivastava 2001; UK Department of Health 1995). Before the report was published in 1995, the current sensible drinking message in the UK was that men should drink no more than 21 units (168 g) and women 14 units (112 g) per week (Department of Health 1992). However this sensible drinking message was open to misinterpretation, as an individual could consume all of their units in a single day or session, and so greatly increase the harmful effects of acute alcohol

consumption. Therefore the benefit of alcohol consumption preventing CHD would be lost.

The responsible daily alcohol consumption guidelines allows for individuals to consume alcohol on a daily basis and if within the responsible drinking guidelines no alcohol free days are required (UK Department of Health 1995). However the recommendation regarding alcohol free days has now changed, and within a report published by the Scottish Government, it is recommended that there are at least two alcohol free days within any week (Catto 2008).

1.2.3 Sessional or "Binge" Drinking

The definition of sessional drinking in the UK is "drinking more than double the recommended daily limit on any one day in the past week", which would equate to 8 UK units (64 g) for men and 6 units (48 g) for women (Catto 2008; Gill, Murdoch, and O'May 2009). This type of drinking pattern is also known as "binge drinking". Binge drinking is a popular term used in the UK media, for the purpose of this literature review and subsequent data analysis within this thesis, "binge drinking" will be referred to as sessional drinking.

The quantitative definition of sessional drinking varies throughout the world, as does the definition of a standard "drink" (see Appendix 1A). Within a standard drink in the UK there are 8 g of pure ethanol, whereas in the USA a standard drink contains 12 g of pure ethanol. The table in Appendix 1A details the variability in sessional drinking definitions throughout the world and also the difference in pure ethanol content within a "standard" drink.

Sessional drinking is referred to as heavy drinking over an evening or similar time period, and is also referred to as heavy episodic drinking (HED) (DiGuarde 2009). HED is a popular term in the United States, but not so widely used in the UK. The amount of alcohol consumed in a sessional drinking episode can be less than a bottle of wine, which is equal to 72 g of alcohol or 9 units of (12 %ABV) wine.

Jayne et al. 2008 describes European countries including the UK as "dry", where beer and spirits are drinks which are well established and alcohol consumed is lower

overall but is more likely to result in intoxication. In comparison in “wet” countries, most commonly Mediterranean counties, where wine is a customary drink and alcohol consumption is high but does not lead to intoxication and is part of the social culture (Jayne et al. 2008). It is also important to note that in “dry” countries beer is a more established drink in comparison to wine and the growth in new beverages, such as “alcopops” or designers drinks (RTDs) has become very popular. The cultural and social problems associated with sessional drinking have been well documented in the media and by the UK Government (Catto 2008; NHS Scotland 2009). However the pathophysiological consequences of sessional drinking have not been well documented. The consequences of the consumption of acute volumes of alcohol over short periods of time has not been fully investigated and compared to the known damaging health effects of chronic alcohol consumption.

1.3 ALCOHOL METABOLISM

1.3.1 Alcohol

Alcohol is a depressant that is absorbed into the bloodstream and distributed to all organ systems in the body. Alcohol in varying doses can cause: staggering, slurred speech, double vision, mood swings, unconsciousness, persistent impotence, loss of libido, hepatitis, oesophagitis and pancreatitis (Agarwal and Srivastava 2001). Heavy or sessional drinking can cause death by respiratory depression. Long term alcohol use has been linked to increased liver disease, heart disease, peptic ulcers, cancers, complicated pregnancies, birth defects and brain damage (Patussi et al. 2010).

1.3.2 Alcohol Metabolism

The metabolism of ethanol is performed by multiple forms of the enzyme alcohol dehydrogenase (ADH) (Lieber 2004). The ADH enzyme exists in the cytosol and catalyzes the conversion of ethanol to acetaldehyde, while also reducing NAD^+ to NADH (Lieber 1992; Lieber 1998). In addition the metabolism of ethanol results in the formation of toxic free radicals and produces oxidative stress. Figure 1.1

illustrates the metabolism of ethanol *in vivo,* by the enzyme alcohol dehydrogenase (ADH).

Figure 1.1: Metabolism of ethanol adapted from Lieber (2004)

ADH is a broad spectrum substrate specific enzyme, with the potential to bind to multiple substrates. ADH is not only specific to xenobiotic (foreign substance) metabolism such as alcohol, but also physiological substrates which includes the dehydrogenation of steroids, oxidation of intermediary alcohols of the shunt pathway of mebalonate, metabolism and also ω-oxidation of fatty acids (Bjorkhem 1972)..

Human liver ADH is a zinc metalloenzyme and occurs in multiple molecular forms. ADH has also been discovered in the stomach and upper gastrointestinal tract (GI). Polymorphisms have been identified at two locations within the gene which codes for ADH, these occur at two loci, ADH2 and ADH3, which encode the β and γ subunits of ADH (Bosron et al. 1993). The latest ADH isoenzyme to be discovered is sigma-ADH; which is found in the upper GI and exhibits variability towards certain ethnic races, which includes the Japanese race. Sigma ADH is less active in a large proportion of the Japanese population resulting in the poor metabolism of ethanol being evident in these individuals.

Ethanol oxidation via the ADH pathway results in the production of large amounts of reduction species, which means that hepatocytes have the inability to maintain redox homeostasis (Lieber 1998). The inability to maintain redox homeostasis contributes to metabolic disorders such as hypoglycaemia and hyperlactacidemia (Lieber 1998). The elevated NADH levels promote fatty acid synthesis and blocks lipid oxidation in the mitochondria, which results in the accumulation of fat, resulting in the condition known as ‘fatty liver’. An overview of the ethanol metabolic pathway is shown in figure 1.2.

Also illustrated in figure 1.2, is the second metabolic pathway, by which alcohol can be metabolised, known as the microsomal ethanol oxidising system (MEOS). The MEOS pathway, comes into effect when chronic alcohol consumption has been undertaken, whereby there is a 4-fold increase in the cytochrome P450 enzyme 2EI (CYP2EI) (Tsutsumi et al. 2089). The induction of the CYP2EI enzyme contributes to the metabolic tolerance against ethanol that develops within individuals who are alcohol dependent. This tolerance to alcohol by way of the MEOS pathway, can also induce tolerance to other types of drugs, including, meprobamate (benzodiazepine) and pentobarbital (barbiturate) (Misra et al. 1971).

From the information illustrated in figure 1.2, alcohol has numerous effects on the body, when ingested. The wide ranging consequences, when acetaldehyde has been produced; include the increase in ethanol metabolism and urine production, displacement of redox haemostasis, metabolic abnormalities within methionine synthesis and vitamin deficiency.

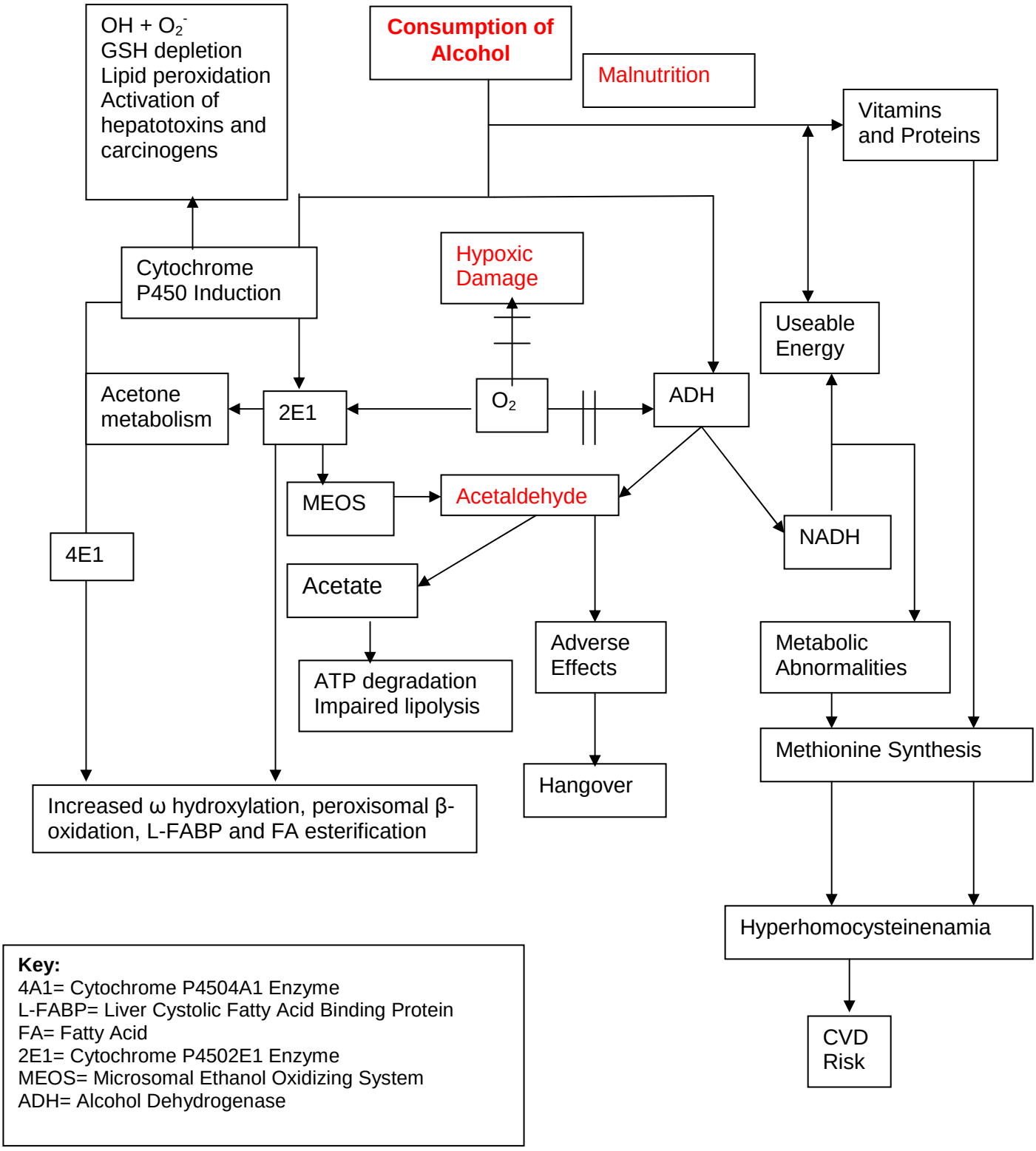

Figure 1.2: Hepatic, nutritional and metabolic abnormalities associated with ethanol consumption adapted from Lieber (2004)

The consumption of alcohol, as outlined in figure 1.2, highlights that alcohol consumption can lead to malnutrition. The vitamins and amino acids most associated with alcohol-induced depletion are folate, vitamins B_6 and B_{12} and the amino acid methionine (Mason and Choi 2005). These vitamin and amino acids play a key role in the metabolism of homocysteine, which has been identified as a biomarker of CVD risk.

Alcohol reduces the bioavailability of the enzyme methionine synthase, which is required for the remethylation of homocysteine to methionine (Lutz et al. 2006). The vitamins folate and vitamin B_{12} are required as co-factors within this reaction. The inhibition of this enzyme due to alcohol consumption inadvertently increases the circulating levels of homocysteine in plasma and serum, which is known to have a direct effect on the cells within the endothelium causing dysfunction and formation of atherogenic plaques (Lutz et al. 2006; Trabetti 2008). Endothelial dysfunction and atherogenic plaques are known risk factors for CVD (Trabetti 2008).

To equate alcohol consumption to potential health risks including risk of CVD, it is important that alcohol consumption is determined accurately and quantitatively. There are various ways to analyse and record alcohol consumption, which include; questionnaires, diaries and biomarkers.

1.4 ALCOHOL CONSUMPTION MEASUREMENT METHODS

The quantitative estimation of alcohol consumption may be performed using a number of methods and/or tools; e.g. alcohol diaries, questionnaires and biological measurement. The most accurate method of estimating alcohol consumption is by measuring the volume of ethanol within breath, blood or urine or by a known biomarker which increases in concentration in the presence of alcohol consumption, such as Carbohydrate-Deficient Transferrin (CDT). This section will describe the different methods for measuring alcohol consumption.

The self-reporting methods used to assess alcohol use, can include: face to face interviews; telephone interviews, diaries and questionnaires (MacDonald 1999). These methods are commonly used within research investigating alcohol

consumption and are a less intrusive way to access alcohol consumption, within a sample of the general population. However these methods listed above do require honesty and accurate memory recall by individuals. Table 1.5 details the different types of questionnaires and diaries which can be used to ascertain self-reporting alcohol use.

Table 1.5: Types of self-reporting methods used for determining alcohol consumption, adapted from MacDonald (1999).

Summary of Drinking	Actual Drinking
Quantity-frequency method	Prospective diaries
Extended quantity-frequency method	Retrospective diaries
Graduated-frequency method	Recall method
Periodic-specific normal week	
Lifetime drinking history	

1.4.1 Questionnaire

A questionnaire is a valuable tool for determining an individual's view point or lifestyle within a particular topic (Boynton and Greenhalgh 2004). The main disadvantages of questionnaires are the reliability of an individual to tell the truth regarding their view point on a chosen subject, this is very relevant when it comes to stating alcohol consumption. A report published recently, has stated that questionnaires and surveys relating to alcohol consumption are known to underestimate the true total consumption, by potentially up to 50% (Catto 2008).

There are a number of different types of questionnaires which can be utilised to measure self-reported alcohol consumption and are listed in table 1.6. The advantages and disadvantages of each type of self reporting questionnaire are also presented in table 1.6.

Table 1.6: Advantages and disadvantages of self-report alcohol use questionnaires.

Questionnaire	Use	Advantage	Disadvantage
Quantity-frequency	Reporting of usual daily consumption of any type of alcohol drink e.g. how often do you drink?	Allows determination of alcohol consumption daily, which can be compared to the UK guidelines.	Does not give alcohol consumption frequency on more than one day.
Extended quantity-frequency	More detailed questions relating to daily alcohol consumption and includes weekends.	More detailed than quantity frequency and takes into account the variability of drinking patterns i.e. is sessional drinking more likely on weekends.	Does not ask questions relating to volume of alcohol consumed.
Graduated frequency	Questions asked in relation to quantity and volume of alcohol consumed.	Frequency of alcohol consumption is estimated in the quantity of alcohol consumed, beginning with largest and scaling down to smallest	Memory recalls potentially a problem, especially if heavy alcohol consumption is being described.
Period-specific normal week	Questions asked in relation to a normal week.	This method allows for consumption to be compared between work days and weekends.	A normal week can vary considerably for certain individuals.
Lifetime drinking history	Asks questions in relation to lifetime of drinking. Can be asked via face-face interview or self-administered questionnaires.	Gives a large amount of data on alcohol consumption over a lifetime period, thus detailed drinking patterns variability during a lifetime.	Memory recall would be a major problem.

Another major group of questionnaires are those employed as screening tools by healthcare professionals.

The AUDIT (Alcohol Use Disorders Identification Test) screening tool emerged from a multicentre WHO collaborative project within six countries and was originally developed to be used within primary healthcare settings (e.g. GP surgeries) (Saunders et al. 1993). It was designed to detect Alcohol Use Disorders (AUDs) (as defined in table 1.3) (Babor et al. 2001). As noted, the AUD categories (hazardous, harmful and alcohol dependence) are associated with increasing levels of risk and harm due to alcohol consumption. An overview of the AUDIT and the associated reliability and validity are summarised in table 1.7.

Table 1.7: Summary of alcohol screening questionnaires, adapted from Burns et al. (2009).

Questionnaire	Reference	No. of Questions	Completion Time	Strength and Limitations	Reliability (Cronbach's α)
AUDIT-C *(Alcohol Use Disorders Identification Test – Consumption)*	(Bush et al. 1998)	3	< 1 minute	Shortened version of the AUDIT questionnaire and therefore designed to be quicker to administer (e.g. advantageous in emergency departments). Designed to detect AUDs and risk drinking. Has been validated in the primary care setting, emergency settings, general population, university student populations and adolescents (Dawson et al. 2005; Harris et al. 2010). Dawson et al. 2005 has suggested different cut-off values for males and females as this has been shown to improve the sensitive and specificity of the AUDIT-C. This has also been suggested within the Bradley et al. (2009) review (Bradley et al. 2009).	0.74-0.94 (Meneses-Gaya et al. 2010; Reinert and Allen 2007)
AUDIT *(Alcohol Use Disorders Identification Test)*	(Saunders et al. 1993)	10	2 minutes	Originally developed by the WHO to detect alcohol dependency in men but has been evaluated in a variety of settings, populations and cultural groups. Original AUDIT development was evaluated in: Australia, Bulgaria, Kenya, Mexico, Norway and the USA (Saunders et al. 1993). AUDIT has established reliability and validity within primary care patients (rural and urban) and subgroups including: women, unemployed, university/college students, adolescents, and different ethnic groups (Dawe et al. 2002; Daeppen et al. 2000). There is a large body of evidence showing high sensitivity and specificity in the aforementioned groups (Reinert and Allen 2007).	Median: 0.83, range: 0.75-0.97 (Reinert and Allen 2007)

Questionnaire	Reference	No. of Questions	Completion Time	Strength and Limitations	Reliability (Cronbach's α)
NET *(Normal drinker, Eye-opener, Tolerance)*	(Bottoms et al. 1989)	3	1 minute	Developed for use in an obstetric population (pre-pregnancy and pregnancy). Shown to be more reliable than CAGE within this population group, but has exhibited lower sensitivity than both the T-ACE and TWEAK. Has not been validated outwith the USA as an independent screening instrument (Burns et al. 2009). Furthermore the literature suggests this questionnaire has only been utilised in one study (Bottoms et al. 1979), therefore explaining the lack of reliability and validity evidence.	Not reported in the literature.
T-ACE *(Take (number of drinks), Annoyed, Cut-down, Eye-opener)*	(Sokol et al. 1989)	4	1 minute	Derivative of the MAST and CAGE questionnaire. Designed for detecting excessive alcohol use in pregnant women. Has an excellent correlation agreement with structured interview in pregnant women for identifying alcohol abuse (Burns et al. 2009). Furthermore, this questionnaire is more reliable in detecting the consumption of high-risk drinking (defined as five or more drinks on one occasion), during pregnancy, compared to CAGE (Burns et al. 2009). Like the NET questionnaire, T-ACE has not been validated outwith the USA as an independent screening instrument (Burns et al. 2009). A recent systematic review by Burns and co-workers has identified only 4 studies (Chang et al. 1998; Russell et al. 1994; Russell et al. 1996; Sokol et al. 1989) where T-ACE has been used as an alcohol screening questionnaire (Burns et al. 2009)	Not reported in the literature.

Questionnaire	Reference	No. of Questions	Completion Time	Strength and Limitations	Reliability (Cronbach's α)
TWEAK *(Tolerance, Worried, Eye-opener, Amnesia, Kut-down)*	(Russell and Bigler 1979)	5	1 minute	Derivative of the MAST and CAGE questionnaire. Designed for detecting excessive alcohol use in pregnant women. Shown to exhibit sensitivity of 75% and 90% within this population (Fiellin et al. 2000). Similar to NET and T-ACE, the TWEAK questionnaire has not been validated outwith the USA as an independent screening instrument (Burns et al. 2009). A recent systematic review by Burns and co-workers has identified only 3 studies (Dawson et al. 2001; Russell et al. 1994; Russell et al. 1996) where TWEAK has been used as an alcohol screening questionnaire (Burns et al. 2009)	0.71 (Coker et al. 2004; Russell and Bigler 1979)
CAGE *(Cut-down, Annoyed, Guilt, Eye-opener)*	(Mayfield et al. 1974)	4	1 minute	Short questions with yes or no answers. Developed for use in men and not considered to be accurate for identifying alcohol abuse in women and different ethnic groups (Bradley et al. 1998). Has exhibited a stronger reliability and accuracy in the in-patient population (medical, surgery and psychiatry) (Dhalla and Kopex 2007). Recent review by Dhalla and Kopex has highlighted the high retest reliability of 0.80-0.95 for CAGE and correlations of 0.48-0.70 with other screening questionnaires (Dhalla and Kopex 2007) However CAGE is of limited value in the primary care population, with the suggested cut off score of ≥2 (Burns et al. 2009).	0.70-0.85 (Dhalla and Kopex 2007)

Questionnaire	Reference	No. of Questions	Completion Time	Strength and Limitations	Reliability (Cronbach's α)
SMAST *(Short Michigan Alcohol Screening Test)*	(Selzer, Vinoku, and van Rooijen 1975)	13	1-2 minutes	Yes or no questionnaires relating to the previous 12 months of alcohol consumption. The shortened version of the MAST questionnaire and therefore is quicker to administer to patients or participants. SMAST has been examined in a primary care setting; however reliability and validity data suggest that the SMAST questionnaire is a better measure of lifetime or past problem drinking in a clinical population (Barry and Fleming 1993).	0.60-0.81 (Gibbs 1983)
MAST *(Michigan Alcohol Screening Test)*	(Selzer 1971)	24	10-15 minutes	Questions with yes or no answers that relate to alcohol consumption within previous 12 months. The first study to determine the reliability of MAST was conducted in 1975 by Selzer and colleagues, which determined that MAST showed a high internal consistency (Selzer et al. 1975). However a study by Crook and colleagues contradicted the coefficients stated in the original reliability study (Crook et al. 1994) and suggested that the MAST questionnaire score be calculated using sub-scales. This would provide more information in terms of alcohol dependence than a single overarching total score. Reliability and validity evidence has shown MAST is more useful in detecting alcohol abuse and dependence in clinical populations, as opposed to the general population (Dawe et al. 2002).	0.83-0.95 (Gibbs 1983; Selzer 1971)

Currently the WHO, NIAAA (National Institute of Alcohol Abuse and Alcoholism), UPSTF (US Preventive Services Task Force) and NICE have repeated the recommendation that the AUDIT is used to screen for alcohol misuse in primary care. Furthermore, they suggest that the AUDIT is more reliable than other screening tools within this setting (National Institute for Health and Clinical Excellence 2010; Rubinsky et al. 2010).

The questions stated within the AUDIT relate to alcohol consumption, drinking behaviour and alcohol-related problems and score an individual out of a total of 40. The higher the total score the more severe the alcohol problem and the likelihood for an individual to develop dependency. The AUDIT scoring criteria are: 1-7 represents low risk drinking; 8-15 is indicative of hazardous drinking (and is appropriate for simple advice focused on reducing consumption); 16-19 is suggestive of harmful drinking (brief counselling and continued monitoring is seen as appropriate) and above 20 is potential dependency (further diagnostic evaluation is warranted) (Babor et al. 2001; National Institute for Health and Clinical Excellence 2010). Babor et al (2001) have reported that a cut-off score of 10 will provide greater specificity, but this will therefore reduce sensitivity and is currently not recommended (National Institute for Health and Clinical Excellence 2010).

There has been a strong body of evidence published in the literature indicating the high sensitivity and specificity demonstrated in the scoring system used within the AUDIT, to detect the criteria of hazardous and harmful alcohol use (Hodgson et al. 2002). For harmful and hazardous alcohol use, 92% of those identified using AUDIT have a score of 8 or above. For those individuals consuming alcohol in a non-hazardous pattern, 94% scored less than 8 (National Institute for Health and Clinical Excellence 2010; Saunders et al. 1993). Since the development of the AUDIT, numerous independent studies have reported that it is a valid and reliable screening questionnaire and is considered to be the gold standard (Hodgson et al. 2002; National Institute for Health and Clinical Excellence 2010; Rubinsky et al. 2010).

Recent findings have suggested that the effectiveness of alcohol screening questionnaires should take into consideration the fact that the diagnostic AUDIT scores for males and females may be different (Aalton et al. 2009). Data published by Aalto and co-workers suggests that the AUDIT cut off score of 8 and above is

suitable for males, but should be lower for females (Aalton et al. 2009). Further work is needed to clarify whether or not the interpretation of AUDIT scores should take into account gender.

The psychometric properties of the AUDIT have been examined within a number of different population samples (Dawe et al. 2002). This has revealed, that with the exception of the geriatric population, the AUDIT has a good internal reliability in all validated population samples. The performance of AUDIT has also been investigated in comparison to other screening questionnaires. For example a comparison with MAST and CAGE showed convergent validity with moderate to high correlations (Dawe et al. 2002). The AUDIT questionnaire has been shown to be consistent in terms of sensitivity in detection of less severe cases of alcohol misuse, in comparison to CAGE and MAST, therefore demonstrating a high sensitivity to detecting non-dependent alcohol misuse (Dawe et al. 2002).

The original AUDIT questionnaire has been shortened to evaluate only alcohol consumption, using three questions and is known as the AUDIT–C (AUDIT-Consumption). It has shown good reliability in detecting heavy drinking and dependency, however the sensitivity and specificity are still lower than the full AUDIT (Bush et al. 1998). Data pertaining to the reliability and validity of the AUDIT-C is highlighted in table 1.7. One major benefit of the AUDIT-C is its appropriate use where time restrictions are in place and under this condition, AUDIT-C has been shown to be reliable (Bush et al. 1998).

The CAGE questionnaire was developed in the USA from a clinical study of medical and surgical patients (Mayfield et al. 1974). CAGE can be administered as a self-reported questionnaire or can be used within an interview, and either mode of administration is reported to have no effect on the patient's response. The reliability and validity of the CAGE questionnaire is summarised in table 1.7. The four questions that form the CAGE questionnaire have exhibited good internal reliability, with all four questions correlating with the other, indicating that the CAGE questionnaire is measuring a single homogeneous construct (Watson et al. 1995). Evidence showing the comparability of CAGE and MAST have shown that CAGE is a better identifier of recent alcohol misuse problems and dependency compared to MAST which has a higher reliability of determining alcohol dependency over an

individual's life time (Dawe et al. 2002). There have been suggestions in the literature that the CAGE should be incorporated into medical history questionnaires, using the question "do you ever drink alcohol?" However if the aim is to detect hazardous or harmful drinking the AUDIT should be used as it is more sensitive (Dawe et al. 2002).

The Michigan Alcohol Screening Test (MAST) was developed for use as a structured questionnaire to detect alcoholism that could be used by a range of clinicians, within different fields (Selzer 1971). The MAST questionnaire has also been developed into a shortened version, which includes 13 questions, compared to the full 24 questionnaire version, known as SMAST (Short Michigan Alcohol Screening Test). The MAST questionnaire was originally developed using a small sample of males who were alcohol dependent alongside a control group. The lack of a range in individuals within the original group used to develop the MAST questionnaire, has not prevented the questionnaire being widely used and showing good reliability and validity. The validity and reliability of both the MAST and SMAST are reviewed in tale 1.7. The cut-off score for MAST is 13, which will detect the presence of the DSM defined alcohol abuse and dependency (Selzer 1971). Using this score MAST has a sensitivity of 0.91 and specificity of 0.76, when detecting alcohol dependency (Ross et al. 1990). Within the original reliability study the author also suggests the score of 5 can reliably determine hazardous and harmful dependency using MAST (Selzer 1971). However more recent evidence has shown the AUDIT is more reliable for the detection of hazardous and harmful drinking (National Institute for Health and Clinical Excellence 2010).

The NET, TWEAK and T-ACE questionnaires have been developed for the use within a specific population: pregnant women. The reliability and validity of NET, TWEAK AND T-ACE are reviewed in table 1.7. Literature evidence suggested that the MAST and CAGE questionnaire did not provide suitable sensitivity within samples of pregnant women, requiring the need to develop screening tools specifically for that sample group (Dawe et al. 2002). The T-ACE questionnaire has been derived from the CAGE questionnaire, where the questions have been reduced from four to three, where the question about guilt has been replaced with a tolerance question (Bradley et al. 1998). Furthermore the NET questionnaire is similar to the T-ACE questionnaire, but replaces the 'cut-down' and 'annoyed'

questions with a question asking if the patient is a normal drinker (Bradley et al. 1998). The TWEAK questionnaire is similar to T-ACE where it merges questions from both the MAST and CAGE questionnaires (Bradley et al. 1998). In a recently published systematic review (Burns et al. 2009) the T-ACE and TWEAK exhibited higher sensitivity (90-92%) and specificity (94-97%) in comparison to NET (sensitivity 71% and specificity 86%). The recently published systematic review by Burns and co-workers suggests that TWEAK and T-ACE, along with AUDIT-C are valid tools for determining at risk drinking in pregnant women, however more work is required to fully validate these tools within this sample and determine the test-retest reliability.

The validity and reliability of alcohol screening questionnaires, as reported in the literature, clearly highlight their ability to detect hazardous and harmful alcohol consumption and dependency (National Institute for Health and Clinical Excellence 2010; Rumpf et al. 202; Saunders et al. 1993). However evidence suggests that their reported reliability and validity (reviewed in table 1.7), may be specific to certain healthcare settings, ages, ethnic and gender groups and this should be taken into consideration before implementing their use (Fiellin et al. 2000).

All the aforementioned screening questionnaires do not ask for grams or units of alcohol consumed when drinking and these questionnaires have been primarily designed to detect alcohol misuse and/or dependency. The AUDIT questionnaire does ask a patient how many drinks they consume in a typical drinking day, however no standard measure of a “drink” is given and as an example, the grams of alcohol found in a large glass of wine (24 g) compared to a measure of spirit (8 g) are very different, but both are defined as a “drink”. Therefore the ability of these questionnaires to over or underestimate alcohol consumption is clear.

Alcohol screening questionnaires, including AUDIT and CAGE have been compared to alcohol biomarkers (e.g. CDT), with aim to compare their reliability and determine the efficiency of both tools in identifying alcohol dependence and/or misuse. A search of the literature has identified several publications which compare alcohol screening questionnaires to biological markers (Comasco et al. 2009; Couture et al. 2010; Hermansson et al. 2003; Miller et al. 2004; Neumann et al. 2009). A study by Couture et al. (2010) compared AUDIT and MAST to a range of alcohol biomarkers,

including CDT, GGT, AST, ALT and MCV in a sample of drink-drive offenders in Canada. The aim of the study was to predict if either tool could determine if the individual was likely to reoffend. Within the sample (N=154), MAST and AUDIT identified 97.9% and 66.0% prevalence of alcohol misuse. However the use of the various alcohol biomarkers did not identify a high prevalence of alcohol misuse within the sample. This could be explained, by the time of blood sampling as the study authors did not take into consideration the half-life of the alcohol biomarkers and depending on the timing of blood sampling the levels of the biomarker could have declined to that of a level below the positive threshold. Therefore not reflecting the drinking time-frame captured by the questionnaire. This would be especially important if a study participant was a sessional drinker, with periods of high alcohol consumption and alcohol free days.

Furthermore a number of studies have shown that both alcohol screening questionnaires (e.g. AUDIT and CAGE), used in conjunction with biomarkers (e.g. CDT) have been successful in determining alcohol dependence (Comasco et al. 2009; Hermansson et al. 2003; Neumann et al. 2009). The study by Hermansson et al. (2003) showed a 20% prevalence of alcohol dependence in the study sample (N=990), detected using either CDT or AUDIT. However no correlation between AUDIT and CDT was demonstrated by Hermansson et al. (2003), when both tools were used together, and this was further demonstrated by Neumann et al. (2009). A mechanism of explanation for no correlation between these two tools lies within what each tool aims to detect. The AUDIT and CAGE screening questionnaires are designed to detect alcohol dependency or problem drinking within a general population. Whereas alcohol biomarkers are designed to detect if drinking has taken place within a recent time frame (CDT detects heavy drinking within a 2 week period), either by way of blood alcohol or breath alcohol and furthermore is used to monitor alcohol abstinence in alcohol dependent populations. The alcohol biomarkers in common usage, including CDT are not actively used to diagnose dependence, which could explain the poor correlation between the two tools and further emphasises the different uses of each tool (Jeppsson et al. 2007).

The most recent alcohol consumption survey to take place in Scotland was the Scottish Health Survey 2003 (Bromley et al. 2003). This nationwide survey asked specific questions relating to alcohol consumption, frequency and type of beverage.

The survey also included selected questions from the CAGE questionnaire and over 13 questions specifically relating to the frequency, type and location of alcohol consumption. A survey conducted by University of Aberdeen and NHS Grampian known as "The Grampian Lifestyle Survey" specifically looked at alcohol consumption in 16-24 year olds (University of Aberdeen 2009). The Office of National Statistics (ONS) also incorporates alcohol consumption into their Good Household Survey (Goddard 2001).

In many general consumption questionnaires; including ones issued by the ONS (Goddard 2001), respondents are asked to equate their alcohol consumption to a given volume of alcohol. An example of this is where the alcoholic drink is measured as follows, one unit is equal to: one glass of wine, half a pint of beer or a measure of spirit. This information given to respondents is actually inaccurate and as a result is underestimating a respondent's true alcohol consumption. To equate one unit to a glass of wine, is underestimating the true unit measurement. A glass of 12% wine measuring 250 ml actually contains three UK units (24 g) of alcohol. The underestimation of alcohol intake from questionnaires results in dangerous alcohol drinking patterns, such as sessional drinking being underestimated (Catto 2008; Gill and Donaghy 2004; MacAskill et al. 2008).

1.4.2 Diary

Diaries have been in use in a number of studies to recall alcohol and/or food consumption (Dawson 2003). Diet and/or alcohol diaries are a common tool within the nutrition and dietetics field to ascertain the diet history of individuals over a prescribed period of time (Gregory et al. 1995). The two different types of diaries employed to determine self-reported alcohol consumption are listed in table 1.5.

One of the main negative issues associated with the use of a diary is the honesty and accuracy of the respondent. A retrospective diary requires the participant to work back in time and remember how much, and which type of alcohol beverage they consumed up to a week ago, which can be problematic for some individuals. However the use of a prospective diary reduces the need to remember when alcohol was consumed and what volume, as this type of diary is completed on a daily basis.

The use of technology has now enabled alcohol diary software to be functional on mobile phones and personal digital assistants (PDAs), which allows the alcohol consumption data to be entered into the program as soon as a beverage has been consumed. A recently published study in the United States of America (USA) has employed this technology whereby a PDA handheld computer was given to student participants to enter their daily alcohol consumption. This allowed the data to be downloaded onto a central computer (Bernhardt et al. 2007). This system has the potential benefit of reducing the likelihood of data being lost as might be the case, if recorded in a paper diary. Additionally data can be analysed on a daily basis, e.g. after an episode of sessional drinking, to quickly ascertain the volume and type of alcohol consumed.

Recording alcohol consumption within a prospective or retrospective diary requires accuracy of recall (Dawson 2003). For example if an individual undertook an episode of sessional drinking on a weekend, and left it until Monday to complete the diary then due to the effects of alcohol on the body, the accuracy of recall of that individual would be less than someone who had just one small glass of wine. The report “How much is Scotland drinking” investigated the self-reporting of alcohol consumption and concluded that people tend to underestimate their true alcohol consumption (Catto 2008). A study published by Perrine et al. 1997 undertook an observational study to investigate alcohol consumption recording, when the participants were observed in a public bar settling and the subsequent self reports. The study found that the self-reported alcohol consumption was significantly lowered than that of the observed data.

A search of the literature found only one journal paper, which assessed the use of AUDIT, quantity/frequency questionnaire and one week alcohol diary and their reliability and sensitivity (Shakeshaft et al. 1998). The study published in Australia in 1998, found that of the three tools listed above, AUDIT was the most sensitive in detecting, what the study referred to as “binge drinkers”, however the definition of binge was not given. The study found similar results when comparing the quantity frequency questionnaire and alcohol diaries and also between the quantity frequency question and AUDIT. No correlation was found between the AUDIT and one week drinking diaries (Shakeshaft et al. 1998). The study by Shakeshaft et al. (1998) did conclude that the most appropriate tool to use was dependent on the

study design and what data was required, as a 1 week drinking dairy can produce more information on drinking pattern, type and frequency in comparison to the other tools used.

Following on from the Perrine et al. (1997) study, it is also important to note diary entries, which state alcohol was consumed in a bar or club, as opposed to alcohol consumed and poured in the home, which are known to differ in volume (MacAskill et al. 2008). Due to the standardised measures available within licensed premises, the accuracy of a poured drink within a licensed premise is going to be more accurate than that of a drink poured at home. A study by Gill and O'May (2007) investigated if drinks poured at home were of a greater volume compared with measures usually served in licensed premises. Study participants were asked to pour a glass of wine or measure of spirit which they would usually have at home. The results of the study showed that drinks which were self-poured were of a greater volume than what would be served within a licensed premise (Gill and O'May 2007). This is an important point to consider when analysing alcohol diaries for consumption. Furthermore the analysis of alcohol consumption within the diary itself requires thought. Determination of the alcohol consumption can be calculated manually from drinks stated provided there is standard definition of measures (e.g. 25 ml spirit shot or 250 ml for a large glass of wine) and an example of a unit calculator can be obtained from the Drink Aware website (Drinkaware.co.uk 2008). The unit calculator provided on the Drink Aware website does not include the full spectrum of all alcoholic drinks available in the UK, and the development of an alcohol drink database or spreadsheet would therefore improve the accuracy of alcohol diary analysis. The use of dietary software, which is commercially available, could also be used to analyse alcohol diaries, however a literature search has not identified any studies using this method. The WinDiets software, developed in Scotland (WinDiets Research Version, Univation Ltd), has the potential to be used for alcohol analysis, however brand names are not included in the database and alcohol measurements, including the %ABV of each drink is not given. Alcohol consumption analysis generated from the use of the WinDiets program, requires caution and the data generated compared to a more detailed tool, such as the unit calculator on Drink Aware Website. A search of the literature has proved no evidence on the reliability or validity of WinDiets for the assessment of alcohol consumption. An alternative method, not involving individual recall and analysis of

questionnaires and/or diaries, is the use of biological markers to assess if alcohol has been consumed.

1.4.3 Biological Alcohol Detection

Biological markers can be measured within a number of body fluids including; blood, breath, hair, sweat or urine. There are a number of biological markers for alcohol consumption that are currently in use. These so called 'biomarkers', are used for clinical diagnosis of alcoholism and also used within the forensic field to ascertain alcohol involvement in criminal activity (Morini et al. 2009). An accurate, sensitive and specific biomarker is of great importance, as this will enable a quantifiable estimation for alcohol consumption in individuals who are consuming alcohol in patterns which can be acute or chronic and over a varying length of time.

1.4.3.1 Biomarkers of Alcohol Consumption

A biomarker of recent drinking patterns would be a major research advance. Currently blood alcohol concentration (BAC) is the standard biomarker for recent alcohol consumption, however it can only reflect alcohol intoxication within a relatively short preceding period. Ethanol can be identified accurately in body fluids or vapours by chemical and enzymatic methods. Ethanol is short lived in the body with complex metabolism and distribution which can vary between individuals causing the relationship between ethanol concentration consumed and that measured in body fluids to be inaccurate (Das et al. 2004; Das et al. 2008, Swift 2003). Blood, urine, breath, hair and sweat are used to identify ethanol *in vivo* but there is controversy over the accuracy (Das et al. 2008; Morini et al. 2009).

Common laboratory tests used for individuals abusing (drinking in a harmful pattern and above responsible drinking guidelines, see table 1.1) alcohol include: patients with liver conditions which present with hyperbilirubinaemia (increase in serum bilirubin levels, both unconjugated and conjugated) and is more prevalent in alcohol dependent patients (Ahlgren et al. 1988; Das et al. 2008; Das et al. 2003; Wadstein and Skude 1979), plasma urate levels can also be used as they correlate with recent alcohol intake (Das et al. 2008). Heavy drinkers are found to have slightly

raised serum alkaline phosphatase (Chalmers et al. 1981) and urea concentration can also be measured as the levels reduce as alcohol inhibits the enzymes in the urea cycle (Paton 1994). A review of the literature by Das et al. (2008) stated the following as common alcohol consumption biomarkers: Gamma-glutamyltransferase (GGT); Mean corpuscular volume (MCV); Platelet Monoamine Oxidase Type B (MAO-B), Transaminases; AST to platelet ratio index (APRI); Albumin and globulin; Acetaldehyde; Haemoglobin associated acetaldehyde (HAA); Dehydrogenases; Lipid profile; Total serum sialic acid; Plasma sialic acid index of apolioprotein J (ApoJ); Carbohydrate Deficient Transferrin (CDT); Serum beta hexosaminidase; Hyaluronic acid; Ethyl glucuronide; Blood alcohol concentration (BAC); Type IV collagen; Cytokines and the Oxidant-antioxidant system. The sensitive and specificity of the aforementioned biomarkers are shown in table 1.8.

Table 1.8: Sensitivity and specificity of alcohol consumption biomarkers as reviewed by Hannuksela et al. 2007

Biomarker	Sensitivity (%)	Specificity (%)
Ethanol	0-100	100
Urinary 5-HTOL/5-HIAA	0-100	>90
Serum GGT	34-85	11-95
Urinary GGT	71	81
MCV	34-89	26-96
AST	15-69	Low
ALT	18-58	Low
Serum CDT	39-94	82-100
Serum TSA	48-82	18-96
Plasma SIJ	90-92	100
Serum β-HEX	69-94	69-98
Urinary β-HEX	81-85	84-96
EtG	Not detectable	Not detectable
PEth	97-99	100
EtS	Not detectable	Not detectable
Hair FAEE	89-100	90
Urinary dolichol	9-68	96
Platelet MAO-B	Not detectable	Not detectable

However a more detailed investigation into the literature has shown the following biomarkers are the most accurate markers of assessing alcohol consumption: carbohydrate-deficient transferrin (CDT); gamma-glutamyltransferase (GGT); ethyl glucuronide (EtG) (in hair and plasma); Phosphatidyl ethanol (PEth); Fatty Acid Ethy Esters (FAEEs); aspirate aminotransferase (AST); alanine aminotransferase (ALT) and mean corpuscular volume (MCV). The sensitivity and specificity of the alcohol biomarkers shown in table 1.8, have changed since the review by Hannuksela et al. (2007) and specifically relates to Ethyl Glucuronide (EtG). A recently published paper by Morini et al. (2009), which examined the use of EtG in hair for the detection of chronic alcohol consumption, showed that it had a sensitivity up to 89% and specificity of 100%. The paper also suggested that EtG measured in hair, was a good biomarker of heavy alcohol use as CDT. However, EtG has not been investigated as thoroughly as CDT (Fleming et al. 2009; Morini et al. 2009). Similar evidence has been suggested for the use of Ethyl Sulphate (EtS), however it has not been investigated as in depth as EtG (Hoiseth et al. 2008). Both EtS and EtG are described as direct ethanol metabolites, and they are produced as a by-product of ethanol metabolism and do not readily exist normally within the body (Hoiseth et al. 2008). CDT is different from EtG and EtS, as the transferrin protein is present *in vivo* and is altered in the presence of alcohol

These aforementioned biomarkers with the exception of CDT are affected by physiological and pathological conditions and also have low sensitivity and specificity (Morini et al. 2009), as shown in table 1.8. CDT has been selected as the primary biomarker for alcohol consumption by the WHO and International Federation of Clinical Chemistry (IFCC), due to the large volume of published peer-reviewed research, which determines CDT as the primary focus biomarker for monitoring alcohol consumption during alcohol abstinence (Jeppsson et al. 2007; Oberrauch et al. 2008).

1.4.3.2 Carbohydrate-Deficient Transferrin (CDT)

CDT is a biomarker used for identifying recent and regular alcohol consumption and can also be used for monitoring abstinence in patients undergoing detoxification and rehabilitation (Helander et al. 2003). Elevated levels of CDT in serum appear after 2

weeks of consuming >50-80 g/day of alcohol, which can equate to 6-10 units or 1 bottle of 12% wine per day (Jeppsson et al. 2007). CDT levels can remain elevated for more than one week after consumption has stopped (Helander et al. 1996; Jeppsson et al. 2007). The use of CDT as a predictor of consumption within samples of healthy individuals who consume alcohol in a sessional pattern has yet to be determined.

1.4.3.2.1 Biochemistry of CDT

Transferrin is a globular protein which transports iron in the plasma (Aisen and Listowsky 1980; Das et al. 2008; Putnam 1975). Transferrin consists of a single polypeptide chain consisting of 679 amino acids arranged in two independent metal ion binding globular domains, known as the N-terminal and the C-terminal. The N-terminal combines the amino acids sequence 1-336 and the C-terminal combines amino acid sequence 337-679. Transferrin also has two N-linked complex glycan chains positioned at 413 and 611 (MacGillivray et al. 1983). The N-glycan complexes are the carbohydrate portion of transferrin. The two transferrin N-glycan chains differ in their branching structure by showing bi, tri or tetra antennary structures. Each antenna ends with a sialic acid molecule which is negatively charged. This results in asialotransferrin and the various sialylated forms monosialo right through to octasialotransferrin, are identifiable in serum (van Noort, de Jong, and van Eijik 1994). The carbohydrate part of the transferrin molecule only represents 6% of the total transferrin molecule (Kamboh and Ferrell 1987). Transferrin reversibly binds polycations which include: iron, copper, zinc, cobalt and calcium, however it is the binding of iron and copper which is of physiological significance. A transferrin molecule can bind with two atoms of ferric ions and an anion, which is usually a bicarbonate ion and this process requires a constant pH (Ehrenberg and Laurell 1955; Schade et al. 1949). The two N-glycan structures bind a maximum of six sialic acid residues. *In vivo* the mean half-life for CDT is 14-17 days (Morini et al. 2009). See figure 1.3, which illustrates the differences between each of the gylcoforms which form CDT.

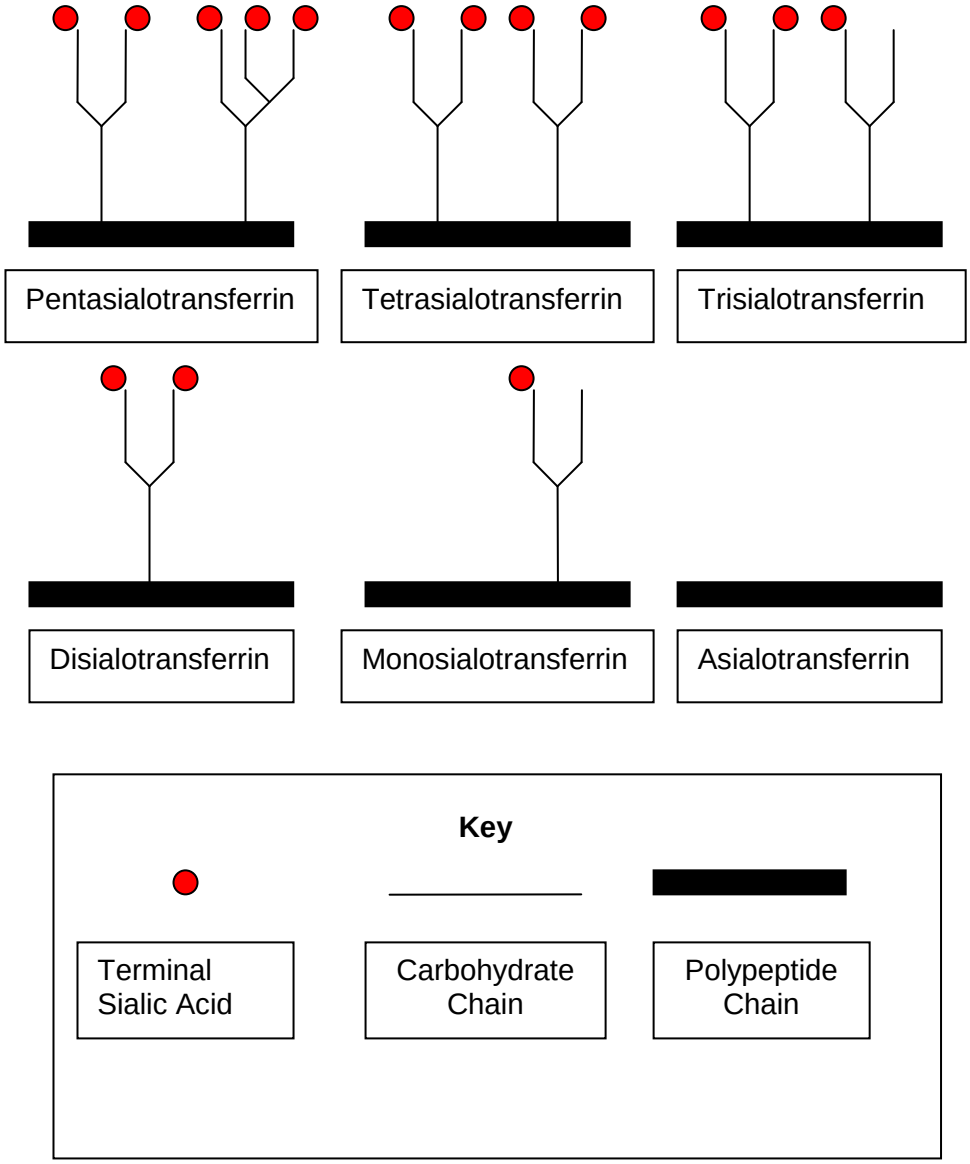

Figure 1.3: Glycoforms of CDT

Alcohol promotes the absorption of ferric iron from the GI tract, potentially by inducing the gastric secretion of hydrochloric acid (HCI) (Charlton et al. 1964). Transferrin is important as it is required for iron homeostasis. It attaches to the receptor on the surface of the cells before iron is released into the cell. Post alcohol consumption causes an increase in iron levels to occur but a decrease in the transferrin synthesis, this results in an in-balance in iron homeostasis, which will increase iron levels, without corresponding increases in transferrin.

1.4.3.2.2 CDT as a Biomarker of Alcohol Consumption

CDT has been approved by the FDA as a clinical diagnostic test for the detection of heavy alcohol consumption (Anton et al. 2001). CDT is the term used to refer to the glycoforms of transferrin which are deficient in sialic acid residues; known as asiolotransferrin, monosialotransferrin and disialotransferrin (Stibler 1991). Recent published literature has confimed that disialotransferrin is the key glycoform altered by excessive alcohol consumption (Jeppsson et al. 2007)

It was in 1970 that Stibler showed abnormal transferrin glycoform patterns in serum and cerebrospinal fluid in relation to alcohol misuse. Stibler used the technique of isoelectric focusing, where the specific glycoforms associated with alcohol showed isoelectric points at or above pH 5.7 (Jeppsson et al. 2007). When the sample was iron saturated, the alcohol affected glycoforms were shown to possess lower sialic acid content in comparison with the major glycoform tetrasialotransferrin, this resulted in the name CDT (Jeppsson et al. 2007; Stibler 1991).

Alcohol consumption can affect a number of enzymes, which includes enzymes responsible for CDT synthesis. Possible enzymes which can be affected are sialy transferase and plasma sialidase which can be responsible for increasing the intracellular level of CDT. A change in the protein transport during the post-translational modification could be a primary mechanism in the impairment of protein metabolism associated with chronic alcohol dependency (Das and Vasudevan 2004).

Patients with liver disease can have normal CDT levels (Behrens et al. 1988; Stibler 1991). However advanced chronic liver disease, primary biliary cirrhosis, chronic active hepatitis and drug induced hepatic insufficiency causes false positive CDT results (Stibler 1991). This can be problematic as a high proportion of alcohol dependent patients have advanced cirrhosis, which means detailed investigation into patient's medical histories is required before a final diagnosis of alcohol use via CDT results is made. A suggestion has been made in the literature that in these cases the result determined by CDT be confirmed with the use of another alcohol biomarker, such as GGT before a diagnosis of alcohol dependence is confirmed (Bianchi et al. 2008).

CDT has high sensitivity (39-94%) and specificity (82-100%) for chronic heavy drinkers and can distinguish them from abstainers or light social drinker (Allen et al. 1994; Behrens et al. 1988; Sillanaukee 1996). The accuracy of CDT is decreased: in individuals who consume lower levels of alcohol and can be classified as responsible drinkers (UK guidelines) (Grönbaek et al. 1995); young alcoholics (Huseby et al. 1997) and women (La Grange et al. 1994; Stibler et al. 1988). However there have been few studies which have examined the potential of CDT as a marker of alcohol consumption in sessional drinkers.

A gender difference, affects which transferrin glycoforms are increased by alcohol, but further work is required to investigate this gender differentiation (Das et al. 2008). No categorical pattern of alcohol consumption, apart from chronic alcohol dependency, has been shown to cause an absolute elevation of CDT in serum (Sillanaukee 1996).

Transferrin is a steroid responsive protein which suggests that sex hormones could contribute to variations in CDT levels. Varying changes in plasma hormones levels induced by conditions such as pregnancy, contraceptives, menstrual cycle and menopause can alter iron homeostasis in women, resulting in a change in CDT levels (Das et al. 2008). The levels of CDT can also be affected by the condition, carbohydrate-deficient glycoprotein (CDG) syndrome. CDG is a group of autosomal recessive diseases, where there is a carbohydrate defect in the serum transferrin. There are two variants, transferrin-B and transferrin-D, where both can interfere in CDT analysis. False negative results can occur due to the genetic D-variants of transferrin and also by primary biliary cirrhosis, hepatocellular carcinoma, viral liver cirrhosis, pancreas and kidney transplantation and also by the drugs which are used to treat these conditions (Sillanaukee et al. 2001). The interpretation of CDT levels in relation to alcohol consumption can therefore require a thorough background investigation of an individual's medical history.

The interpretation of CDT by clinicians needs careful consideration. When CDT values are reported it is important for the testing laboratory to report not only the CDT level but also the cut off level, the reference range and the method employed. This is important as there are several methods used to detect CDT in serum, which

include High Pressure Liquid Chromatography (HPLC), Capillary Electrophoresis (CE) and immunoassays. All of these methods have different reference ranges and cut-off values for a positive result. It is therefore important, when reporting CDT results to state the method used.

1.4.3.2.3 Standardization of CDT

Due to these varying analytical methodologies, a need for standardization of a CDT method is required. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) established a working group for the standardization of CDT (WG-CDT) (Jeppsson et al. 2007). The tasks of the WG-CDT were to define and validate a specific target analyte of CDT, select a suitable reference method, produce reference materials and establish the clinical usage of CDT.

CDT was originally defined as the sum of asialo, monosialo and disialotransferrin. However later studies have shown disialotransferrin and asialotransferrin are missing terminal sialic-acid residues as well as one complete N-glycan in the case of disialotransferrin. Both N-glyans are missing within asialotransferrin (Jeppsson et al. 2007). Monosialotransferrin is not a suitable target glycoform for CDT determination as it is associated with high trisialotransferrin, within the sample and not specifically related to the levels of alcohol consumed (Jeppsson et al. 2007). However asialotransferrin and disialotransferrin are clearly related to alcohol consumption but do have different sensitivity and specificity (Jeppsson et al. 2007).

Asialotransferrin is not detectable in abstinent or social drinkers using current methods, but does become detectable in chronic heavy drinkers (Jeppsson et al. 2007). In chronic heavy drinkers disialotransferrin is detectable and asialotransferrin accompanies this (Jeppsson et al. 2007). The diagnostic sensitivity of asialotransferrin is lower compared with disialotransferrin and too low when detecting chronic to moderate alcohol use. This has resulted in disialotransferrin becoming the current glycoform for CDT detection and will be known as the primary target molecule (Jeppsson et al. 2007).

1.4.3.2.4 CDT Laboratory Methodology

The CDT methodologies in use at present rely on differences in charge (isoelectric point) between disialotransferrin and the other glycoforms (Jeppsson et al. 2007). The measurement of CDT should be obtained after complete iron saturation of the sample, as this influences the isoelectric point and chemical structure, all of which are specific to the method being used. The WG-CDT have suggested that the gold standard method for CDT analysis would be Liquid Chromatography-Mass Spectrometry (LC-MS), however this method is still in development (Oberrauch et al. 2008). At present the reference method for CDT analysis is HPLC (Helander et al. 2003). HPLC can provide specific iron transferrin detection at 460-470 nm with low interference risk; separation of transferrin glycoforms; quantification of disialotransferrin by measuring peak height or area under the curve, which is illustrated on a HPLC chromatograph (Jeppsson et al. 2007). The current reference method was published by Helander et al. (2003) and following that publication, commercial HPLC kits have become available for HPLC CDT analysis by Bio-Rad.

The two other methodologies available for CDT detection are Capillary Electrophoresis (CE) and immunoassays (e.g. N-Latex) (Delanghe and De Buyzere 2009; Delanghe et al. 2007). The CE method is an unspecific, measurement of the peptide bond at 200 nm. This wavelength is not specific for transferrin, which can cause interference with C-reactive protein. This method also has a lower sensitivity than HPLC. However, new methods are being developed. Currently Kings College in London use Capillary Electrophoresis for detection of CDT using the SEBIA capillary system (Kings College Hospital 2007).

The third method for CDT detection is the immunoassay technique. There have been various immunoassay techniques, however, the latest technique and the most common in use now, is the Dade Behring N-Latex CDT immunoassay (Delanghe et al. 2007). The N-Latex assay is the first direct immunoassay where there is no need for separation of the transferrin glycoforms on a mini-column prior to the immunoassay (Delanghe et al. 2007). The function of the N-Latex immunoassay is by way of the antibody mAb, which recognizes the transferrin glycoforms which lack one or both entire N-glycans. This means that the N-Latex assay only recognises

the following alcohol affected glycoforms; mono, asialo and disalo-transferrin (Delanghe et al. 2007).

At present CDT results can be expressed as mg/l, U/l or as a percentage of total CDT within the sample. The reference range for %CDT is 0.8-6%, which encompasses all laboratory methods which are currently in use. CDT is expressed as a percentage as this includes the percentage of bound CDT glycoforms within the serum sample. However, each method does have a specific reference range or cut-off value. The variation of expression of CDT results is causing confusion for researchers and clinicians when diagnosing alcohol dependence. This is a major reason for the need of a standard method for CDT analysis which can be used globally.

1.5 CARDIOVASCULAR DISEASE AND ALCOHOL CONSUMPTION

The detrimental effects of heavy alcohol consumption have been well documented (British Medical Association Board of Science 2008; Catto 2008), however clinical and epidemiological evidence have suggested light to moderate drinking is associated with a reduced risk of CHD; total and ischemic stroke and total mortality in men aged greater than forty years of age and post-menopausal women (Agarwal and Srivastava 2001; DiCasteinuovo et al. 2009; Kloner and Rezkalla 2007; Lakshman et al. 2009). The effect of alcohol consumption on CVD is independent of many other known and suspected risk factors which include smoking, diet, obesity and socioecominc status (Meister et al. 2000; Panagiotakos et al. 2005).

The key mechanisms underlying this protective effect are: an increase in HDL cholesterol levels, decrease in platelet aggregation via inhibition of prostaglandins synthesis and changes in fibrinogen, tissue-plasminogen activator (t-PA) and plasminogen-activator inhibitor (PAI)-1 levels, improvement of endothelial function, reduction of inflammation and promotion of antioxidant effects (DiCasteinuovo et al. 2009; Kloner and Rezkalla 2007; Lakshman et al. 2009; Song 2009). The key mechanisms described above are all involved in the reduction of major CVD events (myocardial infarction, stroke and atherosclerosis) however the exact mechanism of action has yet to be determined (DiCasteinuovo et al. 2009). Studies have shown

an inverse relationship between HDL to CAD risk, where HDL binds with cholesterol in the tissues and may aid in preventing tissue oxidation by LDL cholesterol (Klatsky 2010). The effect of this action by HDL reduces plaque build up in the major arteries, including the coronary arteries (Klatsky 2010). Alcohol has been shown to inhibit the development of atherosclerotic lesions, via dose-dependent mechanisms, in clinical and animal studies (De Gaetano et al. 2002; Haskell et al. 1984; Linn et al. 1993; Renaud and De Lorgeril 1992; Rimm et al. 1999). Recently published literature suggests that the relationship between alcohol consumption and the beneficial effects on CHD are genetic and specifically related to the metabolism of alcohol via the alcohol degrading enzymes (Tolstrup et al. 2009), however this requires further investigation.

A meta-analysis of 30 cohort studies have shown that the consumption of half a measure of a standard drink (24-28 g/day for men and 12-14 g/day for women) per day was enough to show a positive effect on reducing CHD risk (Agarwal 2002; Agarwal and Srivastava 2001; Meister et al. 2000). The meta-analysis also highlighted that there was no increased beneficial effect of consuming more than one standard drink on CHD risk (Meister et al. 2000). Recent literature has stated that the dose of alcohol required to be cardioprotective is one or two drinks per day for men and only one drink per day for women (Lakshman et al. 2009). This recommendation was made on a drink being defined as approximately 14 g of ethanol, which is equivalent to 12 oz of beer (375 ml), 5 oz or wine (150 ml) or 1.5 oz (45 ml) of 80-proof spirit (Lakshman et al. 2009). It is important to note that this drink definition is almost double the ethanol content of one alcohol drink in the UK (Lakshman et al. 2009). A standard unit in the UK contains 8 g of ethanol, which is equated to 83 ml of 12% wine, 25 ml of spirit or half a pint of 3.6% beer.

The relationship between alcohol and CVD can be described by a J or U shaped curve, which can be explained in the following terms: risk is higher when alcohol consumption is high, lower when alcohol consumption is low or moderate; however the risk increases within individuals who abstain from alcohol consumption (Agarwal and Srivastava 2001; DiCasteinuovo et al. 2009; Sander et al. 2005). Figure 1.4 represents a graphical interpretation of a J and U-shaped curve in terms of alcohol providing a cardioprotective effect

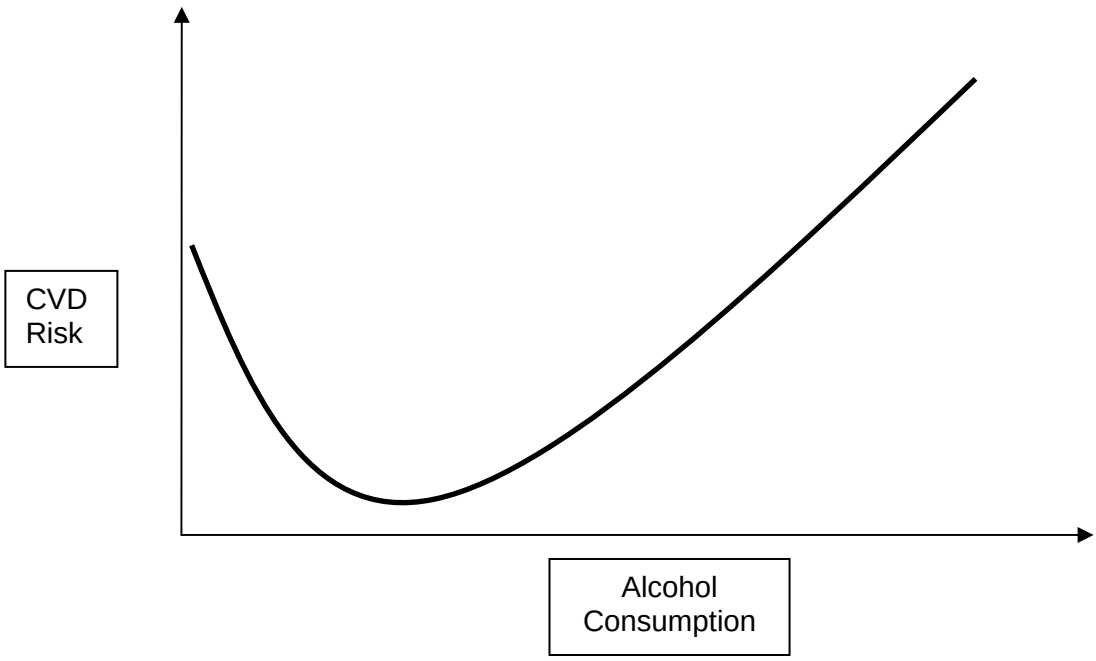

Figure 1.4: Graphical interpretation of a J shaped curve. Adapted from Sander et al. 2005

A review by Rimm et al. 1996 investigated 12 ecological (Artaud-Wild et al. 1993; Criqui and Ringel 1994; Hegsted and Ausman 1988; LaPorte and Cauley 1981; LaPorte et al. 1980; Leger et al. 1979; Nanji 1985; Nanji and French 1986; Renaud and De Lorgeril 1992; Schmidt and Popham 1981; Werth 1980), 3 case-control (Hennekens et al. 1979; Kaufman et al. 1985; Rosenberg et al. 1981) and 10 cohort studies (Farchi et al. 1992; Friedman and Kimball 1986; Gronbaek et al. 1995; Kagen et al. 1981; Kittner et al. 1983; Klatsky and Armstrong 1992; Klatsky and Armstrong 1993; Klatsky et al. 1986; Klatsky et al. 1990; Kozararevic et al. 1980; Rimm et al. 1991; Salonen et al. 1983; Stampfer et al. 1988; Yano et al. 1977) where strong evidence was reported showing moderate alcohol consumption was linked to lower risk of coronary heart disease. It is important to note that in the literature the use of the phrase “moderate” or “light” drinking does not accurately refer to the exact measure of alcohol required to induce these beneficial effects (Andreasson 1998). In terms of a public health messages, it is important to move away from using terms such as “moderate” to describe beneficial alcohol drinking and more towards actual standard measures in either UK units or grams of alcohol.

Recent studies have tried to ascertain the risk of CVD with other lifestyle factors such as diet and exercise, while also taking into consideration the consumption of alcohol (Lakshman et al. 2009; Panagiotakos et al. 2005). The consumption of saturated fat has been well documented to cause an increase in CVD risk and major CHD events, however this was not found in France, and has been termed the 'French Paradox' (Lakshman et al. 2009). In France there is a high consumption rate of red wine, which is known to have a high content of antioxidants and flavonoids which are known to reduce oxidative stress, and thereby reduce endothelial dysfunction (Lakshman et al. 2009). In several Mediterranean countries, it is normal to consume alcohol with a meal, as opposed to in the UK where most alcohol consumption takes place socially and not around meal times (Measham and Østergaard 2009). Red wine consumption in several countries has shown inverse correlations to CHD mortality (Lakshman et al. 2009) and a clinical trial investigating the effects of grape extract showed a beneficial effect on the susceptibility of LDL oxidation in heavy smokers, who were at risk of CVD (Vigna et al. 2003). This evidence suggests that the type of alcohol and the pattern of consumption may have an influence on the ability of alcohol to act as a protective factor against CVD. Studies have shown that drinking wine with meals provided the greatest health effects by preventing the development of atheromatous lesions, but also highlighted that sessional drinking was associated with a higher risk of CHD (DeJong et al. 2003; DiCasteinuovo et al. 2009; Kauhanen et al. 1997; McElduff and Dobson 1997; Ursini et al. 1998). The evidence in the published literature does suggest that alcohol can have a beneficial effect on the cardiovascular system however, it is important to note that the exact volume of alcohol known to be cardioprotective has yet to be established.

Alcohol consumption has been linked to increased blood levels of the amino acid homocysteine, which is an independent risk factor for CVD (Antoniades et al. 2009; Refsum et al. 1998). Alcohol raises homocysteine levels in two ways, firstly by inhibiting the conversion of homocysteine to another amino acid, methionine and secondly as a consequence of alcohol-induced vitamin deficiencies, altering its metabolism (Bleich et al. 2000a). It is this accumulation of homocysteine, which causes atherosclerosis by a direct effect on arterial tissue resulting in increased risk of CHD, stroke and MI (McCully 2001). As excessive alcohol consumption and sessional drinking are associated with adverse cardiovascular risk, the

measurement of homocysteine can give an insight into the potential risk of developing cardiovascular disease (Song 2009).

1.6 HOMOCYSTEINE

1.6.1 Introduction

Homocysteine is a non-protein forming, sulfhydryl containing amino acid and is an intermediate in the remethylation of the amino acid, methionine (Vinukonda 2008). The chemical structure is shown in figure 1.5.

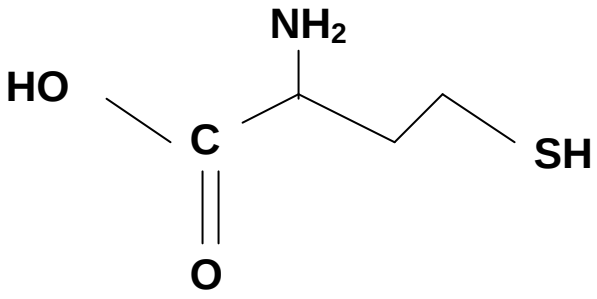

Figure 1.5: Chemical Structure of Homocysteine (Jakubowski 2006)

The metabolism of homocysteine can occur in two ways; either using the transsulphuration pathway or the remethylation cycle. Homocysteine can be transsulphurated to cystathionine or remethylated to methionine, and these reactions are controlled by enzyme action. Methionine is regenerated by the remethylation pathway under the action of methionine synthase (MS). These reactions are dependent on the presence of methylenetetrahydrofolate (MTHF) which is controlled by the enzyme methylenetetrahydrofolate reductase (MTHFR) and the co-factors tetrahydrofolate (folate) and methylcobalamine (vitamin B_{12}). The remethylation and transsulphuration of homocysteine from methionine, in terms of chemical structure is shown in figure 1.6.

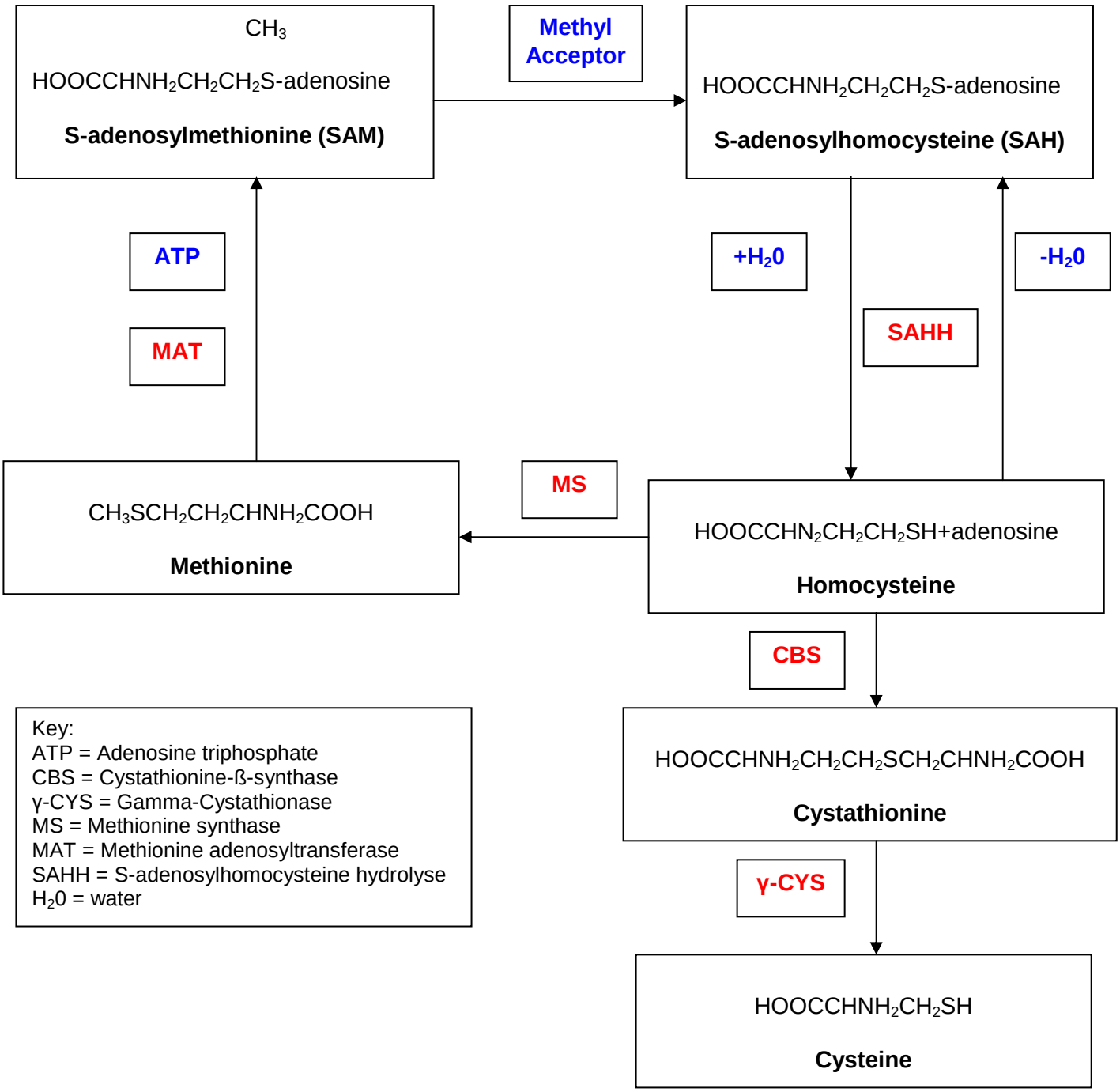

Figure 1.6: Remethylation and transsulphuration of homocysteine in terms of chemical structure. Adapted from McCully (2001).

In 1962 the first homocysteine metabolism abnormality was reported in mentally retarded children due to frequent thromboembolic events. Seven years later it was McCully (McCully and Wilson 1975) who descibed the vascular pathology of

homocysteinuria, followed by Wilcken and Wilcken 1976 who showed the frequent association between abnormal plasma homocysteine and coronary disease. The importance of the pathophysiological actions of homocysteine is illustrated in the following lists of conditions: neural tube defects, pregnancy complications, mental disorders, cognitive functions, cancer and cardiovascular disease.

1.6.2 Biochemistry of Homocysteine

The remethylation of homocysteine to methionine is dependent on methionine being available in the diet. Methionine is an essential amino acid, found in meat, milk and eggs (Trabetti 2008). During the remethylation cycle, homocysteine gains a methyl group from 5-methylenetetrahydrofolate (5-MTHF) to form methionine, as catalysed by methionine synthase. This reaction requires vitamin B_{12} and folate, in their biologically active forms; tetrahydrofolate and methylcobalamine and are referred to as cofactors of the reaction. This reaction occurs in all tissues. When this reaction occurs in the liver, as methionine is highly active within this organ, another enzyme becomes involved; betaine-homocysteine-methyltransferase (BHMT). It acts as a methyl donor (Trabetti 2008). Methionine is metabolised into s-adenosylmethionine (SAM) by the enzyme methionine adenosyltransferase (MAT). The function of SAM is to act as a methyl donor, where the loss of two methyl groups from SAM results in the production of S-adenosylhomocysteine (SAH). From SAH, homocysteine is hydrolysed in a reversible reaction by the enzyme S-adenosylhomocysteine (SAHH). There is competition between SAM and SAH at different binding sites which can inhibit the methylation (Trabetti 2008).

The second metabolic pathway that is relevant to the biochemistry of homocysteine is transsulphuration. In this pathway homocysteine condenses with serine to produce cystathionine, by the enzyme cystathionine ß-synthase (CBS). Cysthathionine is then hydrolysed to cysteine by the enzyme γ-cystathionase (CYS) (Trabetti 2008). Both these reactions require vitamin B_6 in the biological form; pyrioxidal 5'-phosphate (Trabetti 2008). This reaction only occurs if there is an excess of dietary methionine, as the enzyme methionine synthase becomes inhibited, thus activating the transsulphuration pathway (Trabetti 2008). Figure 1.7 demonstrates the biochemistry of homocysteine.

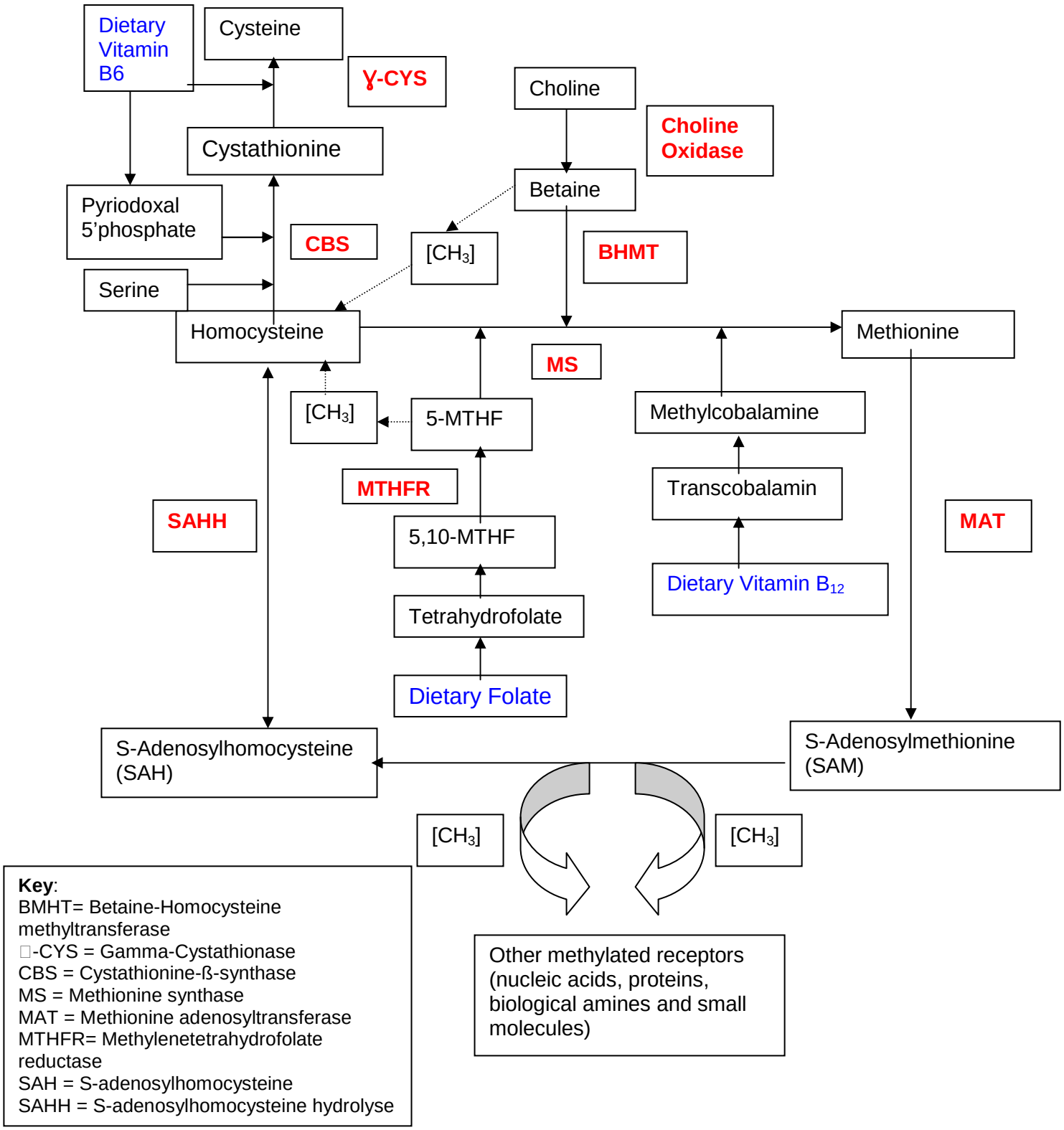

Figure 1.7: Biochemistry of Homocysteine (adapted from Kharbanda and Barak 2005 and Vinukonda 2008)

Under normal physiological conditions homocysteine formation and degradation are balanced; where 50% is remethylated to methionine and the excess homocysteine is moved into the circulation (Hankey and Eikelboom 1999). In the circulation <1% of homocysteine is present in its reduced form (Trabetti 2008). Approximately 10-20% of total homocysteine is present as homocysteine-cysteine mixed disulphide and homocystine (a dimer of homocysteine) (McCully 2001). The remaining 80-90% of homocysteine is protein bound (Trabetti 2008).

1.6.3 History of Homocysteine Discovery

Homocysteine was discovered in 1932, by the American biochemist Vincent DuVigneaud (DuVigneaud 1952). The discovery was made by removing the methyl group of methionine, which indicated that homocysteine was an intermediate of methionine metabolism. Homocysteine has the same functional group as the amino acid cysteine; specifically sulphydryl, amino and carboxyl groups, but has one additional carbon atom, thus naming it homocysteine (DuVigneaud 1952). The four carbon backbone of homocysteine enables formation of an internal anhydride called homocysteine thiolactone. Homocysteine thiolactone is a five member ring which forms stable salts with strong acids, but when neutralised with weak bases, the ring of bonds opens resulting in the formation of two peptide bonds between two molecules of the thiolactone and produces the compound homocysteine diketopiperazine. Homocysteine can be produced from homocysteine thiolactone by hydrolysis with strong bases.

It was not until 1962 that the significance of homocysteine was realised. Children with mental retardation, accelerated growth and propensity to thrombosis of the arteries and veins were found to excrete homocysteine in urine, a condition known as homocystinuria (McCully 2001). The significance of homocysteine in this condition was first realised by the American biochemist Kilmer S. McCully, who spent his career developing the theory of homocysteine.

McCully studied a number of cases of homocystinuria in children who died suddenly. The first case he studied was an archived case from 1933, where an 8 year old boy who was found to have widespread arteriosclerotic plaques and thrombosis of the

carotid artery and death was caused by stroke. McCully hypothesised that homocystinuria was due to a deficiency of the enzyme cystathionine synthase, a pyridoxal phosphate dependant enzyme (McCully 2001). McCully investigated a second case study of homocystinuria, in 1968, eight years after the initial significance of homocysteine was realised, which showed that homocystinuria was caused by deficiency in the enzyme methionine synthase which is a folate and vitamin B_{12} dependent enzyme. This case identified the important potential of excess homocysteine to cause atherogenic plaques. The 1968 case was of a 2-month old baby boy who had advanced atherogenic plaques in arteries that supplied blood to the major organs throughout his body. From the two case studies that McCully analysed, he concluded that due to differences in the enzymes involved, homocysteine caused atherosclerosis by a direct effect on the cells and tissues of the arteries (McCully 2001). The conclusion by McCully was furthered due to the discovery in later years that homocystinuria was also caused by a deficiency in the enzyme methylenetetrahydrofolate reductase (MTHFR), which is shown in figure 1.7.

Further investigations into the condition, homocystinuria did not occur until 1962 when investigators in Northern Ireland, began screening the urine of children with mental retardation for the presence of amino acids using newly developed techniques of paper and column chromatography (McCully 2001). Several children were found to have homocysteine present in their urine, and were diagnosed with homocystinuria. Children with homocystinuria were found not only to be mentally retarded but also have accelerated growth, dislocated ocular lenses, osteoporosis and a tendency to develop thrombosis in arteries and veins (McCully 2001). Mudd and co-workers at the National Institutes of Health discovered cystathionine synthase, the vitamin B_6 dependent enzyme, which is a co-factor for homocysteine to cystathionine metabolism and was deficient in many cases (McCully 2001). Following on from the research conducted by Mudd, Spaeth and co-workers in Philadelphia discovered that vitamin B_6 supplementation was an effective treatment for homocystinuria (McCully 2001) as this was involved in the transsulphuration pathway, shown in figure 1.7.

1.6.4 Procedures to Consider when Measuring Homocysteine in Body Fluids

At physiological pH, homocysteine in its reduced form exists in only trace amounts *in vivo,* whereas most homocysteine occurs in various disulfide forms (Ducros et al. 2002). Around 70% of homocysteine is bound to albumin, as either protein-bound homocysteine or homocysteine-albumin mixed disulfide (Ducros et al. 2002). The remaining 30% exists as mixed disulfides with other thiols. The most abundant is cysteine-homocysteine disulfide (Ducros et al. 2002). The storage of whole plasma and serum causes redistribution of plasma thiols so the protein-bound fraction increases at the expense of the free acid soluble fraction (Ducros et al. 2002). Determination of free homocysteine requires immediate blocking of free thiols at the sampling time, which is not convenient for regular daily laboratory practice. Homocysteine is measured as total homocysteine (free and bound) and not free homocysteine (Ueland 1995). The anthropometric and laboratory conditions which can affect homocysteine levels are detailed in table 1.9 and 1.10.

Table 1.9: Conditions which effect homocysteine analysis (Ducros et al. 2002).

Fasting Conditions	Blood sampling should be carried out after a fast of 12 hours, with a light meal the evening before (Rasmussen and Moller 2000). A high protein or low protein diet can effect homocysteine measurements (Refsum et al. 1998; Ueland et al. 1993).
Influence of Age	Plasma homocysteine concentrations are related to age and gender. An age-related increase of plasma homocysteine levels is linked to a decline in glomerular function; as this is known to decrease in functionality with age (Norlund et al. 1998). Also the uptake of B-vitamins declines with age, which influences levels of homocysteine, due to the B-vitamins acting as co-factors within the conversion of methionine to homocysteine.
Influence of Sex	Women have a lower homocysteine concentration than men, up until the menopause. However the influence of sex hormones on homocysteine has not been established (Rasmussen and Moller 2000; Wouters et al. 1995). The possible differences in plasma homocysteine levels between genders could be explained by the higher muscle mass in men and higher creatinine synthesis in men compared to womens due to plasma creatinine and homocysteine concentration being significantly correlated in healthy subjects (Ducros et al. 2002).
Influence of Ethnicity	The differences in homocysteine concentrations in ethnic groups are not consistent (Rasmussen and Moller 2000). However the differences could be explained by the C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) enzyme. There is a variability of this enzyme in ethnic populations, where there is a 10% occurrence within the Caucasian population, in comparison to the African and American populations, where this mutation is almost absent. The $MTHFR_{(C677T)}$ polymorphism causes elevated plasma homocysteine concentrations, regardless of folate and vitamin B_{12} status.

Table 1.10: Laboratory Conditions which effect homocysteine (Ducros et al. 2002)

Posture	During venepuncture, the participant should be sitting in the supine position. Two studies suggest that the position can affect homocysteine concentration (Rasmussen et al. 1999; Thirup and Ekelund 1999).
Anti-coagulant	The determination of homocysteine is carried out on plasma as opposed to serum. Homocysteine is constantly produced in the erythrocytes which is released to the extracellular compartment. This is why a faster centrifugation is required and sampling on dry ice is avoided (Ueland et al. 1993), as the temperature of dry ice will cause the haemolysis of red blood cells, resulting in the inability to produce plasma. It is therefore recommended that whole blood be stored on wet ice. The most commonly used anticoagulant is EDTA (Ducros et al. 2002).
Blood Storage	The intra-erythrocyte concentration of homocysteine has been evaluated at 0.8 µM; a level 10 times less than in plasma (Ubbink et al. 1992). The speed of release of homocysteine from blood cells is nearly constant and is independent of plasma homocysteine concentrations. The storage of whole blood at room temperature, before centrifugation can cause the following increases in plasma homocysteine levels: for up to 1 hour=10%; 4 hours=20-35% and after 24 hours=60-75% (Fiskerstrand et al. 1993; Ueland et al. 1993). However a study by Houze et al. 1999 showed a lower increase in homocysteine concentration at room temperature, where there was a significant increase of 25% between the fourth hour to the tenth hour of storage. When blood samples are drawn into EDTA tubes and stored on ice, total homocysteine is stable for 4 hours (Ducros et al. 2002; Fiskerstrand et al. 1993)
Plasma Storage	After centrifugation, homocysteine is stable for 4 days at room temperature, several weeks at 0-2ºC and years at -20ºC (Israelsson et al. 1993; Ubbink et al. 1992).
Haemolysis	Does not influence plasma homocysteine concentrations and does not increase concentration (Fiskerstrand et al. 1993; Ueland et al. 1993).
Variation	Intra-individual variation range of 7-15% of normal homocysteine plasma concentration (Clarke 1998; Garg et al. 1997).

1.6.5. Homocysteine Analytical Laboratory Methodology

Homocysteine can be detected in a variety of different laboratory techniques, which include: chromatography, electrophoresis, immunoassay and enzymatic assay (detailed in table 1.11).

In regard to the analytical homocysteine chromatography methods the most commonly used methods are: Mass spectrometry (MS) with liquid chromatography (LC) or gas chromatography (GC), high pressure liquid chromatography (HPLC); and ion-exchange liquid chromatography.

1.6.5.1. High Pressure Liquid Chromatography

This is the most commonly used technique. A major advantage of this method is the ability to jointly determine the different plasma thiols compounds along with homocysteine. The HPLC method utalises three different detection techniques, which include; photometric, fluorescence and electrochemical. Overviews of each of the HPLC detection methods are described in table 1.12. The plasma homocysteine HPLC-ED method by Houze et al. (2001) and Cummins (2005) was adapted and employed for the sample analysis within this thesis.

Table 1.11: Analytical methods for homocysteine determination, adapted from Ducros et al. (2002).

Method	Sample Pre-treatment	Plasma Volume (µl)	Upper Limit of Linearity (µmol/l)	CV Inter-assay for mean total homocysteine level (%)	Throughput (per day or hour)
GC-ID-MS	High workload and derivatization	100	30-300	2.6, 5.3, 5.7	96/day; 160/day
LC-MS-MS	High workload	100	60	5.9	40/hour
HPLC-FD	High workload and derizatization Wavelength range 380-510 depending on fluorogenic reagent	60-150 50 (Bio-Rad)	50-300 100 (Bio-Rad)	3.2 4.8 (Bio-Rad)	90/day 150/day (Bio-Rad)
HPLC-ED	High workload Oxidation potential +0.80 volts	60	100	5.6	60/day
IEC	Medium workload	500	100-1000	7.8	25-50/day
FPIA	None	50	45-50	3.1	20/hour (IMX) 60/hour AxSYM
ICL	None	15	50	3.9	150/hour
EIA	Low workload	25	50	6.2	96 tests/2.5 hours
Enzymatic method	Low workload None	100 5	80 100	2.8 3.7	45/hour
CE-LIF	High workload and derivatization	100	200	7.8	100/day

Table 1.12: HPLC detection methods used for the analysis of homocysteine (Ducros et al. 2002).

Detection Method	Overview	Advantages	Disadvantages
Photometric	The sample is reduced by dithiothretiol, separation of thiols by HPLC and post-column derivatization with the 4,4'-dithiodipyridine thiol specific labelling agent and subsequent detection of products by ultraviolet detection at 324 nm. This method gives high precision with an intra-assay of 1.5%, inter-assay of 2.5% and sensitivity of less than 50 nm. This technique is commonly used in laboratories where a fluorescence detector is not available.	High sensitivity.	Laborious method, Modest throughput.
Flurometric	This detection method is the mostly commonly used along with HPLC (Ducros et al. 2002). It involves precolumn derivatization with flurogenic reagents for thiols, which is then followed by HPLC. For this method the mean within-assay CV is 1% and mean between-assay CV is 3.2%. The reagents used with this method must be nonfluorescent, contain no fluorescence impurities and react rapidly and specifically with homocysteine and other thiols to form stable products. Examples are: monobromobimane (mBrB; excitation 380 nm, emission 470 nm), halogenosulfonylbenzofurazans (sBD-F and ABD-F; excitation 380 nm, emission 510 nm) and o-phthaldialdehyde (OPA; excitation 340 nm, emission 450 nm).	High sensitivity, High precision (1.0%; 3.2%).	High throughput.

Detection Method	Overview	Advantages	Disadvantages
Electrochemical	For this detection no derivatization of thiols is required prior to detection. This method does have a higher mean intra and inter assay CV of 3.9% and 5.6% (D'Eramo et al. 1998). The reference method was reported by Malinow et al. 1989 and modified by Smolin and Schneider 1988. The assay for detection of total homocysteine using HPLC-ED involves the reduction of the biological sample with sodium borohydride, separation of thiols by HPLC and detection of non-derivatized thiols with duel mercury and gold amalgam electrodes, which has greater specificity towards sulfhydryl components (Rabenstein and Yamashita 1989). The flaw with the Rabenstein and Yamashita (1989) method is the possible contamination of flow cells and fouling on electrode, causing deterioration, whereby inducing a decrease in the sensitivity of the assay. To combat this problem a gold electrode is now used (Wu et al. 1994). The gold electrode requires less preparation and maintenance, exhibits good sensitivity for thiols and does not use toxic mercury. A commonly and extensively used HPLC-ED method for detecting homocysteine in plasma was published by Houze et al. (2001).	High sensitivity; high specificity; no derivatisation. Full automation.	Precision is less and detector maintenance required.

1.6.6 Homocysteinaemia

Homocysteinaemia is defined as the total amount of all homocysteine forms in the plasma (tHcy) (Hankey and Eikelboom 1999). The plasma total homocysteine concentration may vary greatly, while intracellular levels have a narrow range (Moat et al 2004). Plasma concentrations are dependent on the diet within a given population and can even vary within groups of the same population (Trabetti 2008). The range of concentrations of homocysteine found in human plasma are shown in table 1.13.

Under physiological conditions, 5-20 mmol of homocysteine is synthesized each day (Refsum et al. 1998). A high percentage of homocysteine is converted to cysteine by the enzyme cystathionine β-synthase (CBS) or to methionine. The CBS enzyme regulates plasma homocysteine levels so normal basal concentrations range from 5 to 15 µM with a mean of 10 µM (Ducros et al. 2002). The level of homocysteine in plasma can be indicative of hyperhomocysteinemia, which is outlined in table 1.13. Hyperhomocsyteinemia is defined as the elevation of circulating homocysteine above the normal reference range of 5-15 µmol/l in plasma (Hankey and Eikelboom 1999).

Table 1.13: Plasma concentrations in human plasma (Kang 1995)

Plasma Homocysteine Range	Concentration in Plasma (µmol/l)
Normal	5-15
Moderate hyperhomocysteinaemia	15-30
Intermediate hyperhomocysteinaemia	30-100
Severe hyperhomocysteinaemia	>100

The accumulation of homocysteine can be due to three causes: transsulphuration defects based on cysthathionine ß-synthase deficiency; remethylation defects based on methionine synthase deficiency and MTHFR deficiency (Trabetti 2008). Another confounding factor in the metabolism of homocysteine is the shortage of cofactors: vitamin B_6, B_{12} and folate which can cause elevated levels (Mason and Choi 2005). The transsulphuration and remethylation pathways are shown in figure 1.7.

Elevated plasma homocysteine can be caused by the following:

1. Mutations in enzymes of metabolism mainly methylenetetrahydrofolate (MTHFR) and methionine synthase (MS) (Maron and Loscalzo 2009).
2. Nutritional deficiencies of vitamin co-factors such as B_6, B_{12} and folate (Maron and Loscalzo 2009).
3. Diseases such as chronic renal failure, malignancies, acute lymphoblastic leukaemia, anaemia, hypothyroidism and diabetes (Trabetti 2008).
4. Physiological factors such as age and gender (Jacobsen 2009).
5. Pharmaceutical medications which include: methotrexate, phenytoin, carbamazepine, nitrous oxide, theophylline, metaformin, colestipol, niacin, penicillamina, thiazide and diuretics (Ducros et al. 2002).
6. Lifestyle factors including smoking, coffee, alcohol consumption, alcoholism and physical activity (Ducros et al. 2002).

1.6.7 Pathology Associated with Hyperhomocysteinaemia

As previously noted, hyperhomocysteinaemia has been implicated in the following conditions: vascular damage, cognitive impairment, pregnancy complications, neurologic and psychiatric complication and neoplastic diseases (Trabetti 2008).

There are common pathogenetic mechanisms which can cause vascular injury, and can lead to the manifestation of clinical signs which have been linked with increased homocysteine levels. A review of the literature by Trabetti (2008) has identified the key mechanisms, which are described in table 1.14.

Table 1.14: Key mechanisms of vascular pathogenesis induced by elevated homocysteine (Trabetti 2008).

Mechanism	References
Oxidative damage of the endothelium by the suppression of vasodilator nitric oxide (NO)	(Chambers et al. 2000; Durand et al. 2001; Harker, Harlan, and Ross 1983; Kanani et al. 1999; McCully and Ragsdale 1970; McCully and Wilson 1975; Stamler and Loscalzo 1992; Starkebaum and Harlan 1986; Upchurch et al. 1997; Visioli et al. 2002)
Promotion of platelet activation and aggregation	(Di Minno et al. 1993; Durand et al. 1996; Durand et al. 1997; Harker et al. 1976; Harpel et al. 1996; Stamler et al. 1993; Upchurch et al. 1997)
Alteration of normal procoagulant-anticoagulant balance promoting thrombosis	(Coppola et al. 1997; Fryer et al. 1993; Hajjar et al. 1998; Harpel et al. 1996; Lentz and Sadler 1991; Lentz et al. 1996; Nishinaga et al. 1993; Ratnoff 1968; Rodgers and Conn 1990)
Impaired methylation of homocysteine	(Bilsborough et al. 2003; Jonasson et al. 2003; Leoncini et al. 2003; Stuhhlinger et al. 2003; Upchurch et al. 1996; Wanby et al. 2003)
Vascular smooth muscle cell proliferation	(Majors et al. 1997; Tsai et al. 1994; Wang et al. 1997)

Hyperhomocysteinaemia encourages endothelial oxidative damage and dysfunction by inhibiting nitric oxide (NO) synthase which decreases the bioavailability of NO (Trabetti 2008). Under normal physiological conditions NO detoxifies homocysteine by forming S-nitroso-homocysteine which is a vasodilator. Excess homocysteine is not fully neutralised by NO but is auto-oxidised to homocystine which in turn produces free radicals which are toxic to endothelial cells (Trabetti 2008). Also under normal physiological conditions glutathione neutralises free radicals but elevated homocysteine decreases glutathione peroxidase activity (Trabetti 2008). Another mechanism of endothelial injury is by the reduced catabolism of asymmetric dimethylargnine which acts as a strong inhibitor of NO synthase (Stamler and Loscalzo 1992; Stuhhlinger et al. 2003; Wanby et al. 2003).

Excess homocysteine can be converted to homocysteine thiolactone, which is a thioester of homocysteine. There is an association between homocysteine-

thiolactone and low density lipoprotein (LDL) as it produces atherogenic oxidative damage to the endothelium (Trabetti 2008). A report by Chwatko et al. 2007 has shown homocysteine thiolactone is elevated in patients who are deficient in the enzymes cystathionine ß-synthase and MTHFR.

In patients with hyperhomocysteinaemia, platelets have a normal lifespan of 10 days and morphology. Trabetti et al (2008) has suggested that homocysteine might activate platelets which can increase their aggregation and adhesion. The biosynthesis of platelet thromboxane A_2 is highly increased in individuals with homocysteinuria and the increased production of thromboxane A_2 could be the cause of an increased risk for thrombosis (Trabetti 2008). Homocysteine in plasma is auto-oxidised to produce free oxygen radicals which initiates lipid peroxidation in the endothelial plasma membrane or in lipoproteins (Trabetti 2008). Platelets are activated by oxidised LDL, which is atherogenic. Studies have shown that thrombosis is promoted by homocysteine as it alters the procoagulant-anticoagulant balance due to increasing or decreasing the levels of coagulation factors (Trabetti 2008).

The elevated levels of homocysteine which cause hyperhomocysteinaemia can have a negative impact on DNA methylation (Trabetti 2008). This results in altered gene expression which can affect the endothelial and smooth muscle cells of the vascular wall. Several publications have suggested that elevated levels of homocysteine can induce proliferation of the vascular smooth muscle cells, which can lead to luminal narrowing, whereby reducing blood flow and increasing endothelial dysfunction (Trabetti 2008).

Ross et al. (1999) has suggested that excess homocysteine can induce inflammation and atherosclerosis. Homocysteine activates Nuclear Factor-KappaB (NF-KB) which induces the expression of monocytes chemoattractant protein 1 (MCP-1) and interleukin 8 (Poddar et al. 2001; Wang and O 2001; Wang et al. 2001). The ability of homocysteine to activate monocytes and cause their proliferation will induce the expression of cytokines and the inhibition of the expression of macrophage migration inhibitory factors (Su et al. 2005) and thereby induce an inflammatory response within the endothelium.

A number of lifestyle factors have been shown to elevate plasma homocysteine and cause hyperhomocsyteinemia (Panagiotakos et al. 2005). Alcohol consumption is one such lifestyle factor and published literature has shown that alcohol-dependence can elevate plasma homocysteine levels (Bleich et al. 2000d; Cravo and Camilo 2000), however this has not been shown in healthy individuals who consume alcohol in a range of patterns. Figure 1.8 demonstrates the pathway of hyperhomocysteinemia from cause to effect.

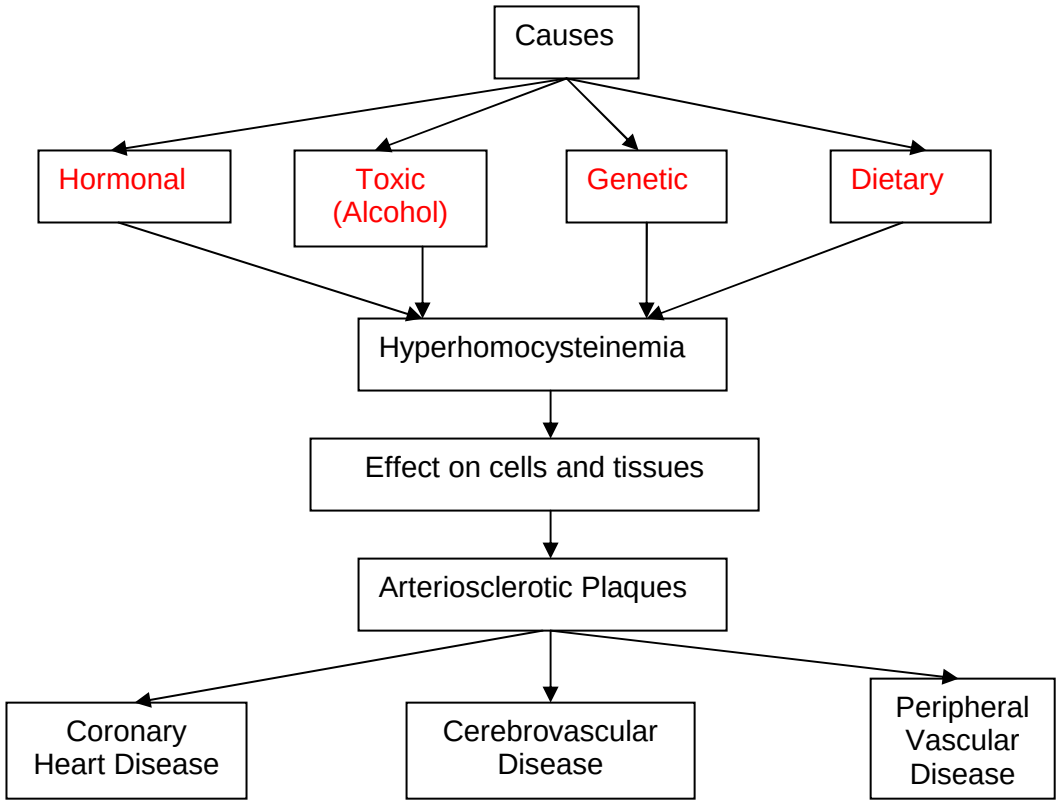

Figure 1.8: Effects of elevated homocysteine adapted from McCully (2001)

1.6.8 Homocysteine and Alcohol Consumption

Alcohol can have detrimental effects on homocysteine metabolism through the following actions: inhibition of methionine synthase by acetaldehyde causing elevation of homocysteine (Lutz et al. 2006). Alcohol consumption also reduces the bioavailability of folate and vitamin B_{12}, which are important co-factors in homocysteine metabolism (Lutz et al. 2006). Chronic alcoholism can induce hyperhomocysteinaemia (>100µmol/l in plasma) (Bleich et al. 2004), however the influence of sessional drinking is less well described (Chiuve et al. 2005). Within the

18-24 yrs age group, which includes students and young professionals, sessional drinking is often the preferred method of consumption (Gill 2002). The possibility that this particular pattern of drinking may, through changes in plasma homocysteine levels affect CVD disease risk profile in the early decades of life, requires investigation.

There have been two studies published in 2008/2009 which investigated the effect of alcohol consumption on homocysteine levels (see appendix 1B). A study by Devika et al. 2008 investigated the effect of alcohol withdrawal in a sample of alcohol dependent individuals. The study by Gibson et al. 2008 conducted an intervention using two different types of alcohol (red wine and vodka) for a two week period and subsequently analysed homocysteine levels at the end of the intervention.

Devika et al. (2008) conducted an alcohol withdrawal study with a sample size of 50. Males and females were recruited into either group one, which was an eight week detoxification or group two (participants did not undergo a detoxification regime). Homocysteine was analysed by ELISA. Results showed that after detoxification, homocysteine levels significantly decreased ($p<0.002$) within group one participants; on admission homocysteine concentration were 26±13.36 µmol/l and after detoxification it had been reduced to 14.31±5.7 µmol/l which is within the reference range. It is important to note that the study participants recruited to this study received folic acid, vitamin B_6 and B_{12} supplementation, during the detoxification regime, which is known to have a reducing effect on homocysteine levels.

The study design adapted by Devika et al. (2008) was similar to a study conducted by Bleich et al. 2000a, in Germany, which employed a shorter detoxification over a period of 3 days. The main difference between these studies was that Bleich did not supplement his participants with folic acid and vitamin B_{12} over the detoxification period, and still produced data which showed homocysteine reducing over time of detoxification (on day one of admission 33.6±25.2 µmol/l; on day three 13.9±8.8 µmol/l) ($p<0.001$). This suggests that without supplementation, plasma homocysteine levels can be reduced to within normal reference levels, once heavy alcohol consumption has ceased. A study by Cravo et al. 1996 investigated homocysteine in an alcohol dependent sample, however unlike Bleich or Devika, the

study which Cravo conducted investigated homocysteine levels with no detoxification or intervention. Cravo found that homocysteine was two times higher in a sample of alcoholic dependent individuals compared to controls and that those who were abusing beer did have significantly lower levels of homocysteine, compared to individuals who were drinking other alcohol beverages heavily (Cravo et al. 1996). This could be due to the higher levels of folate in beer compared to other alcoholic beverages (Bleich et al. 2000c).

Gibson et al. (2008) conducted an alcohol "dosing" study, where males were recruited into two different groups; group one (N=40) consumed only red wine and group two (N=45) consumed only vodka. Participants were asked to consume 24 g (3 UK units) of alcohol daily and were classified as responsible drinkers, according to the UK recommended daily consumption guidelines before undertaking the study (UK Department of Health 1995). Homocysteine was analysed by HPLC-FD. Gibson found that homocysteine levels increased after the two week red wine intervention by 5% ($p=0.03$), but not in the group receiving the vodka intervention. This finding is interesting and not expected as there has been published literature showing the positive effects of moderate red wine consumption on increasing antioxidant status and reducing plasma homocysteine levels (Tsang et al. 2005). The results found by Gibson et al. (2008) were also consistent with published literature, whereby when homocysteine levels increase, folate and vitamin B_{12} decrease (Antoniades et al. 2009; Refsum et al. 1998). The study conducted by Gibson, is reflective of a study conducted by Bleich, which was published in 2001 (Bleich et al. 2001). The study conducted by Bleich was a 6 week alcohol intervention, where participants consumed 30 g of beer, wine or spirits daily. At the end of the 6 week intervention the results showed that homocysteine significantly increased ($p<0.02$) regardless of which alcohol was consumed.

1.6.9 Homocysteine and B-Vitamins

The B-vitamin family are a group of water-soluble vitamins, found in yeast, germ seeds, eggs, liver and vegetables. B-vitamins have varied metabolic functions including growth factors and co-enzymes and are also known as the B-complex.

Vitamin B_{12} and folate are inter-linked through the B_{12}-dependent methionine synthase reaction where homocysteine is converted to methionine by donation of a methyl group from the circulating methytetrahydrofolate to produce tetrahydrolate which is required for thymidine and DNA synthesis. If there is deficiency of vitamin B_{12} or folate then there is a decrease in DNA. A deficiency of DNA can lead to the presence of immature megaloblasts in tissues such as bone marrow and small intestine which have a rapid cellular turnover (Halsted and Keen 1990).

Deficiency of vitamin B_{12} is not common as the recommended daily allowance (RDA) is as low as 1 µg while it is stored in the body to a maximum of 2-3 mg. If dietary intake of vitamin B_{12} is low then stored vitamin B_{12} is sufficient to allow normal function for several years. However the levels of vitamin B_{12} in the serum can often be elevated due to displacement of vitamin B_{12} from the liver and also possibly by increased binding to plasma transcobalamin (Halsted and Keen 1990).

Nutritional deficiencies of the B vitamin family, specifically folate and vitamin B_{12} are often associated with high total homocysteine levels which results in hyperhomocysteinemia. Vitamin B_{12} deficiency may result in considerable hyperhomocysteinemia which is rapidly normalised after replenishment with the specific deficient vitamin (Brattstrom 1996).

Table 1.15: Dietary vitamins and synthetic supplement (Brattstrom 1996)

Dietary Vitamin	Synthetic Supplement
B_{12}	Cyanocobalamin
Folate	Folic acid

The synthetic oral supplement, listed in table 1.15 can be taken by individuals to increase folate and/or vitamin B_{12}. Several studies have shown oral treatment of folic acid (5-10 mg/day) reduces hyperhomocysteinemia by 30-60% (Brattstrom 1996). 1 mg of folic acid and 0.4 mg cyanocobalamin are sufficient therapeutic doses to effectively reduce homocysteine levels (Brattstrom 1996).

1.6.9.1 Folate

Folate in the form of tetrahydrofolate (THF) is a substrate and coenzyme in the acquisition, transport and enzymatic processing, of one-carbon molecules for amino and nucleic acid metabolism and metabolic regulation. Folate donates one-carbon molecules during the methylation of homocysteine to methionine, which is used by S-adenosylmethionine in cellular methylation reactions and protein synthesis. The presence of folate is also key in supporting nucleic acid synthesis as it acts as a carbon donor during the synthesis of purines and thymidylate and during deoxycytoine methylation. The involvement of folate is also important for the interconversion of glycine and serine and during the breakdown reaction of histidine to form glutamate. The normal metabolism of folate is showed in figure 1.9.

Folate and homocysteine levels are affected by gender, where males have approximately 25% higher levels of circulating homocysteine compared to females (Fabris et al. 2009). The $MTHFR_{(C677T)}$ genotype in conjunction with low folate status, strongly affects homocysteine levels in males and to a certain extent in females (Fabris et al. 2009). Published literature has shown that there is strong evidence which suggests that there is a relationship between inadequate folate status and an elevated homocysteine concentration, which leads to the subsequent risk of the following conditions: coronary heart disease, venous thrombosis, carotid artery stenosis, and other cardiovascular diseases (Quinlivan et al. 2006; Stowell et al. 1997).

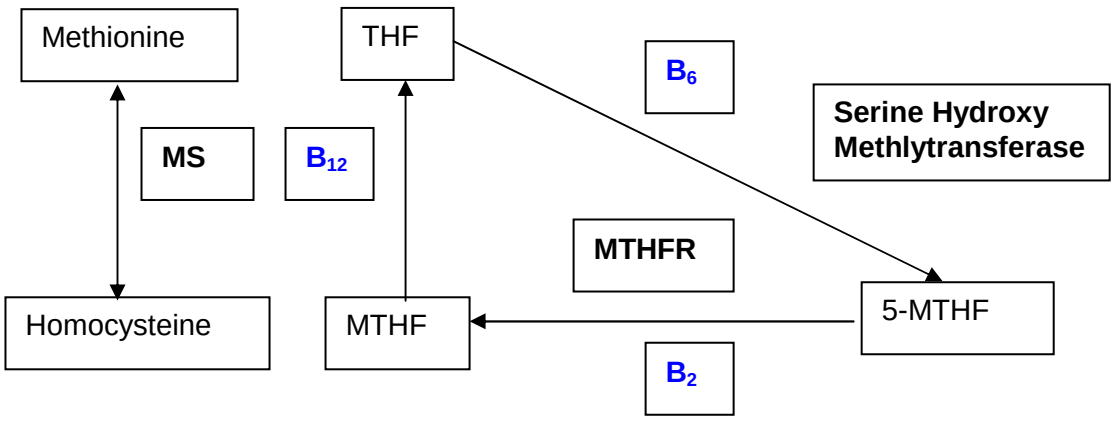

Figure 1.9: Normal metabolism of folate adapted from Mason and Choi (2005).

The ingestion of alcohol lowers serum folate levels and impairs haematological recovery, which is possibly through a catabolic effect of alcohol metabolism on the folate molecule (Halsted and Keen 1990). Low serum folate levels has been shown in more than two thirds of sessional drinkers (Halsted and Keen 1990). A study by Shai et al. 2004 has shown that total homocysteine is inversely associated with dietary folate and B vitamins and positively associated with alcohol consumption. The acute or chronic consumption of alcohol evokes the inhibition of the enzyme MS, as shown in figure 1.10 (Lutz et al. 2006). Folate and vitamin B_{12} are substrates and co-enzymes of this reaction. The inhibition of MS by ethanol reduces the intracellular concentration of methionine and SAM, but increases the circulating concentration of the reaction precursor, homocysteine (Mason and Choi 2005). It is also important to note that alcohol consumption can deplete the levels of circulating folate specifically, highlighting that a sufficient volume of THF will not be present to act as a co-factor within the reaction (Mason and Choi 2005).

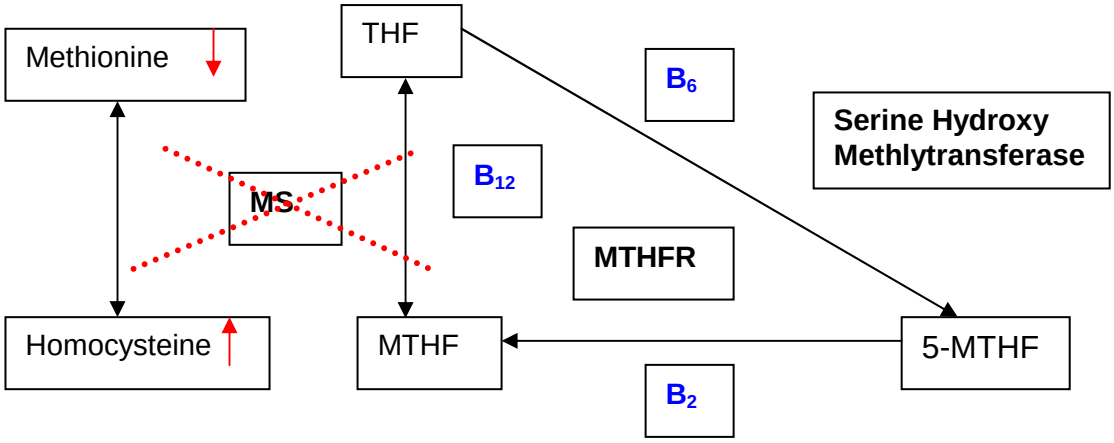

Figure 1.10: Effect of alcohol on the metabolism of folate adapted from Mason and Choi (2005).

1.6.9.2 Vitamin B_{12}

Cobalamin, also known generically as Vitamin B_{12} is an important intermediate in methionine synthesis. Vitamin B_{12} has two important cofactors which play an important role within methionine synthesis, these are methylcobalamin and adenosylcobalamin (Morel et al. 2005). Vitamin B_{12} and its corresponding cofactors are required by methionine synthase (MS) and mitochondrial enzyme

methylmalonyl-CoA mutase for the conversion of methionine to homocysteine (Morel et al. 2005). The metabolism of vitamin B_{12} is shown in figure 1.11. Chronic consumption of alcohol impairs the uptake and rention of vitamin B_{12} by the liver and other peripheral tissues (Cravo and Camilo 2000).

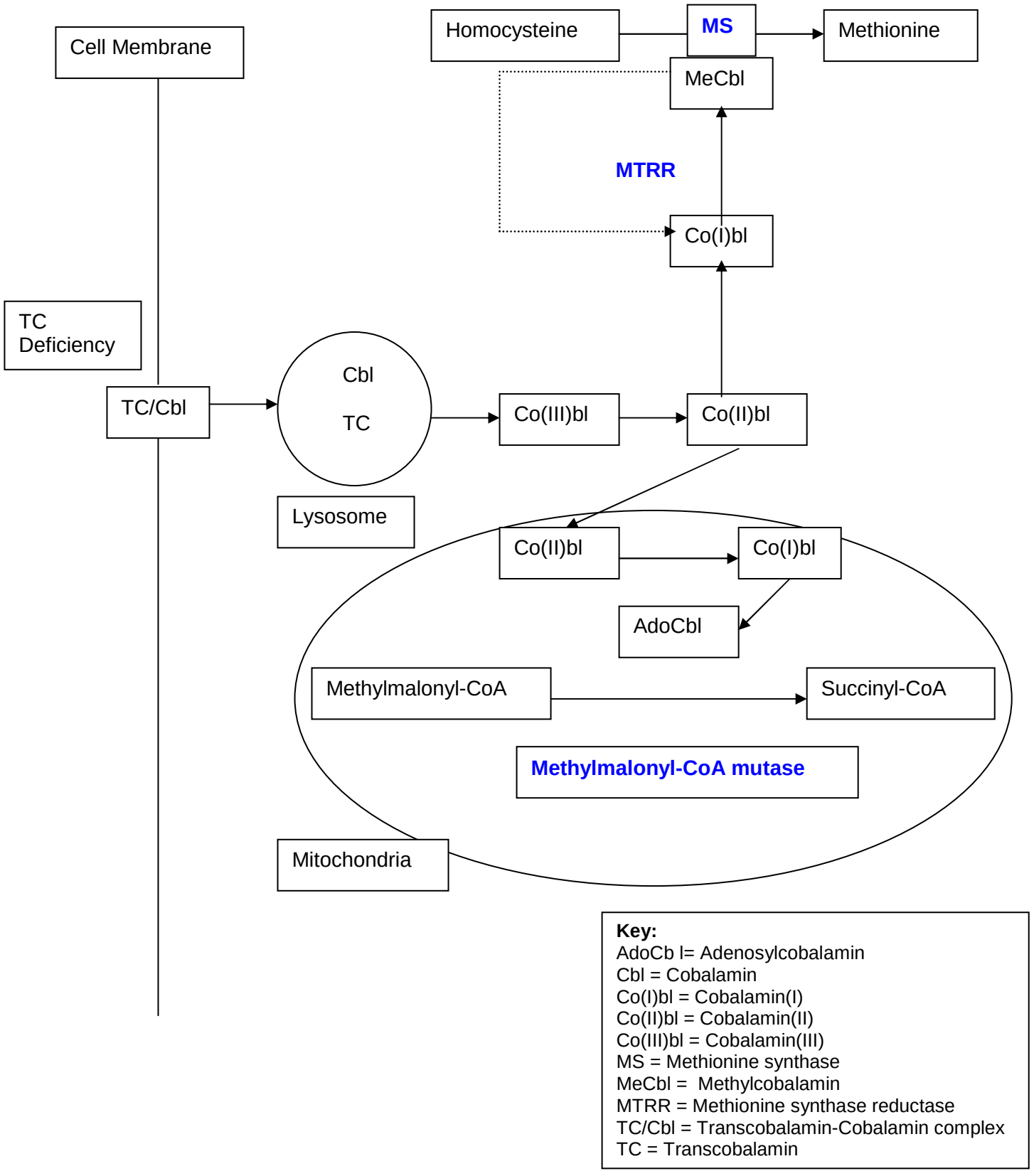

Figure 1.11: Metabolism of Cobalamin (Vitamin B_{12}) adapted from Morel et al. (2005)

Thiol derivatives of cobalamin have been identified, and are known as thiolatocobalamins. An important cobalamin thiol derivative is glutathionylcobalamin (GSCbl) which was identified in 1960 (Birch et al. 2009). GSCbl is more active in promoting the synthesis of methionine via the enzyme methionine synthase. This suggests that thiolactocobalamins could be more effective in treating conditions associated with hyperhomocysteinemia and oxidative stress in comparison to other cobalamins (Birch et al. 2009), therefore reducing homocysteine levels by supplementation.

The recommended daily intake (RDI) of vitamin B_{12} is very low, at 2-3 µg. Vitamin B_{12} is stored within the body for a long period of time, and does not deplete rapidly, which results in the low RDI value (Solomon 2007). Deficiency of vitamin B_{12} can cause megaloblastic anaemia, decline in neurocognitive function, and also adverse effects have been reported on bone formation and immune system response (Kräutler 2005; Solomon 2007).

1.6.9.3 The Relationship between Homocysteine, Folate and Vitamin B_{12}

Several studies were published in 2008 and 2009 which investigated the impact of folate and vitamin B_{12} supplementation on homocysteine status (Appendix 1C).

Pfeiffer et al. (2008), Stea et al. (2008) and Wotherspoon et al. (2008) conducted intervention studies where the diet was supplemented with folate and/or vitamin B_{12}. In the study conducted by Stea et al. (2008), plasma homocysteine decreased by 10% after intervention ($p=0.002$). The change in homocysteine concentration was inversely related to the change in folate levels. A similar study conducted by Wotherspoon et al. (2008), was a randomised control trial (RCT) where participants were randomised to receive; either a 5 mg folate supplement daily or a placebo. Data showed that plasma homocysteine levels were reduced by 25% in the group which received the folate supplement ($p=0.03$), however biomarkers of endothelial function (NO and prostaglandins) showed no difference after supplementation. It should be noted that the participants who were recruited into the Wotherspoon et al. (2008) study were individuals who were diagnosed with type 2 diabetes and microalbuminuria. The results produced from this study, are indicative of this study

sample, which might not be reflected in a healthy sample, such as the sample within the study by Pfeiffer et al. (2008). The article published by Pfeiffer et al (2008) examined the data from The 3rd National Health and Nutrition Examination Survey (NHANES III) survey conducted within the USA before and after folate fortification. The data showed that there was a 10% reduction of homocysteine levels within the USA sample population after folate fortification was introduced in the country.

The above studies suggest that folate supplementation can successfully reduce circulating homocysteine levels and there is an inverse relationship between homocysteine and folate.

Another investigation into the relationship between homocysteine, folate, vitamin B_{12} and CVD was conducted by Dangour et al. (2008). The study was conducted within an older adult population (age was greater than 75 years) and both genders were recruited. The study found that participants, who had homocysteine levels within the highest percentile, had a two times greater risk of all cause mortality in comparison to participants who had homocysteine levels in the lower percentile. The authors found no association between folate and vitamin B_{12} and mortality, but cautioned that this could be due to low statistical power (Dangour et al. 2008).

An assessment of folate and vitamin B_{12} status in adolescents was conducted by Al-Tahan et al. (2008). The study found that homocysteine was higher in males, but vitamin B_{12} was higher in females. Supporting existing literature, Al-Tahan found that the presence of the MTHFR polymorphism increases plasma homocysteine as serum folate decreases (Frosst et al. 1995). The study by Al-Tahan et al. (2008) also links with the publication by Tanaka et al. (2009) who investigated the genetic factors which affect plasma homocysteine, folate and vitamin B_{12}. Tanaka et al. (2009) confirmed by meta-analysis that MTHFR is consistently association with homocysteine status.

In the forgoing work the analysis of folate and vitamin B_{12} were carried out almost exclusively by immunoassay (enzymatic and radioimmunoassay) (Al-Tahan et al. 2008; Dangour et al. 2008; Stea et al. 2008). However alternative methods exist, which include colourmetric assay and chromatography. A study by Mitchell et al. (2009) evaluates a method of liquid chromatography-multiple reaction monitoring-

mass spectrometry (LC-MRM/MS) for identifying folate and homocysteine. The author found that this method produced results with a reduced error and greater statistical power, in comparison with the method mentioned previously (Mitchell et al. 2009). This finding should be considered when choosing a methodology for folate laboratory analysis.

1.6.10 Methylenetetrahydrofolate Reductase (MTHFR)

The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) has a key function in the folate cycle and contributes to homocysteine metabolism (see figure 1.7) (Fabris et al. 2009). The MTHFR enzyme catalyzes irreversibly the reduction of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MTHF) (Fabris et al. 2009). 5-MTHR is the main circulatory form of folate under physiological conditions and acts as a carbon donor during the conversion reaction of homocysteine to methionine (Fabris et al. 2009). Within the MTHFR gene a single nucleotide polymorphism (SNP), located on the 1p36.3 chromosome, has been identified. This SNP has introduced a Cysteine to Thymine transition at the nucleotide 677 (C677T) in exon (a nucleotide sequence in DNA that carries the code for the final mRNA molecule and thus defines a protein's amino acid sequence) four which has resulted in an alanine to valine amino acid exchange which affects the reaction potential of the enzyme (Fabris et al. 2009; Frosst et al. 1995). The amino acid exchange results in a thermo-labile variant of MTHFR, known as t-MTHFR being produced which has a reduced enzyme capacity (Fabris et al. 2009; Frosst et al. 1995). Studies have shown that individuals who posses the TT (mutant homozygote) or CT (heterozygote) genotype have approximately 30% and 65% reduced MTHFR enzyme activity compared to individuals who have the wild-type heterozygote CC genotype (Fabris et al. 2009; Frosst et al. 1995; Kluijtmans et al. 1996). This reduced enzyme activity in the individuals who posses either mutant genotype results in circulating homocysteine level (Fabris et al. 2009), whereby highlighting the importance of the MTHFR enzyme activity and its effect on circulating homocysteine levels. Studies within alcoholic dependent patients have shown a higher prevalence of the MTHFR polymorphism within this group (Lutz et al. 2006). However this has not been investigated in healthy individuals who consume alcohol in a range of non-dependent patterns.

1.6.10.1 The Relationship between Homocysteine and the MTHFR Enzyme

A search of the literature found several studies published since 2008 which directly associate the effect of the methylenetetrahydrofolate (MTHFR) polymorphism on homocysteine status (Appendix 1D).

All studies reported an association between the presence of the MTHFR polymorphism and levels of homocysteine. Alessio et al. (2008) investigated the prevalence of MTHFR and CBS in Brazilian children (N=220) and found that the 677TT, 1298AA and 68WW mutations in the MTHFR gene were associated with an increase in homocysteine concentrations. Folate and vitamin B_{12} were also measured in the sample and were found to not be significantly different in each mutation group. The study concluded that there was a genetic influence which was affecting homocysteine concentrations, independently of vitamin B_{12} and folate levels (Alessio et al. 2008).

Yang et al. (2008) conducted a large scale study (N=6793) of participants who were genotyped from The 3rd National Health and Nutrition Examination Survey (NHANES III) study. Yang found that in participants who carried the MTHFR polymorphism, regardless of ethnicity or race, there was significantly higher plasma homocysteine levels coupled with lower serum folate levels. The study conducted by Yang et al. (2008) also investigated alcohol consumption and found alcohol consumption was significantly associated with serum homocysteine concentrations, in a sample of individuals who consumed alcohol in a range of patterns. The mean serum homocysteine concentration increased from 8.5 µmol/l in abstainers to 9.3 µmol/l within participants who consumed ≥7 drinks per week (p=0.0028). The study participants who were found to carry the $MTHFR_{(C677T)}$ polymorphism had 22% lower serum folate levels and 25.7% higher homocysteine levels compared with an individual who carried the CC genotype. The study also concluded that supplementation with folic acid reduced homocysteine levels in individuals carrying the MTHFR polymorphism ($p<0.001$) (Yang et al. 2008).

The importance of MTHFR in relation to CVD was investigated by Grassi et al. (2008) and Naess et al. (2008). Both studies found that the $MTHFR_{(C677T)}$ polymorphism, when present, caused an increasing effect on homocysteine

concentrations (Grassi et al. 2008; Naess et al. 2008). Naess et al (2008) found that homocysteine levels in men were higher when, MTHFR was present and this could predict onset of venous thromobosis. This association however was not found in women. An important study by Nagele et al. (2008) highlighted the potential effects of anaesthesia on homocysteine levels. Nagele et al (2008) conducted a study with 140 participants who were undergoing a surgical procedure using nitrous oxide (N_2O) as anaesthetic. Participant's blood samples were analysed for homocysteine, folate, vitamin B_{12} and the MTHFR polymorphism. The study found that participants who carried the MTHFR polymorphism C677T or A1298A were at a higher risk of developing increased homocysteine levels after undergoing N_2O anaesthesia ($p=0.03$) (Nagele et al. 2008). This may have important implications from individuals undergoing surgery, especially cardiovascular surgery, where homocysteine may already be elevated.

Husemoen et al. (2009) investigated the relationship between lifestyle factors, homocysteine and the $MTHFR_{(C677T)}$ polymorphism in a 5 year follow up study. All lifestyle factors were investigated including alcohol consumption and the prevalence of the MTHFR polymorphism. Husemoen et al (2009) found that blood levels of homocysteine were not affected by any lifestyle factors including the consumption of alcohol, however in individuals who carried the TT polymorphism there was an inverse relationship between homocysteine and beer consumption. This was not present within CC/CT individuals. The presence of the TT polymorphism is found to make individuals more susceptible to B-vitamins supplementation, which could explain the inverse relationship of homocysteine levels and beer consumption as certain types of beer contain a high level of folate (Cravo and Camilo 2000; Cravo et al. 1996; Husemoen et al. 2009).

1.7. HOMOCYSTEINE AND CARDIOVASCULAR DISEASE RISK

It is widely accepted within the published literature that homocysteine is associated with cardiovascular disease (CVD) risk and is independent of other atherosclerotic risk factors (Lutz et al. 2006; Refsum et al. 2006). Homocysteinaemia causes increased CVD risk due to several mechanisms: increased thrombogenicity; increased oxidative stress; over activation of redox sensitive inflammatory pathways;

impaired endothelial function and atherogensis (Antoniades et al. 2009). Moderate hyperhomocysteinaemia occurs in 20-30% of patients with atherosclerosis (Refsum et al. 1998). Homocysteine measured on admission can predict later on-set of CVD events, including MI, cardiac death and acute coronary syndromes (Antoniades et al. 2009). This suggests that homocysteine is a good CVD risk predictor in a healthy population and also within a population which already has established CVD.

1.7.1 Homocysteine and Potential for Development of Atherosclerosis

Autopsy studies of children dying with homocystinuria in 1964, linked death due to thrombosis of arteries and veins in the major organs. Changes found in arteries were attributed to the atherogenic effect of homocysteine on connective tissues because of molecular similarity with penicillamine (McCully 2001). Penicillamine is similar in its chemical structure to homocysteine and can form mixed disulfides with sulfhydryl agents that have the potential to induce cell injury within the endothelium. The effect of penicillamine has been investigated within the literature and has been shown to not effect the endothelium in terms of cell injury (Wall et al. 1980) In certain cases of homocystinuria, arterial lesions were described as atheromas, but in most reports there was no mention of the relation to atherosclerosis. The tendency of patients with homocysteinuria to develop arterial and venous thrombosis was attributed to increased adhesiveness of platelets (McCully 2001).

Atherosclerotic plaques have been induced in monkey and rat animal models (McCully 2001). In the monkey animal model, the animal was deficient in vitamin B_6, which causes hyperhomocysteinemia due to inhibition of cystathionine synthase, which is the enzyme responsible for the conversion of homocysteine to cystathionine and is the most common deficiency in cases of homocystinuria (McCully 2001). The rat animal model was deficient in choline, folic acid and vitamin B_{12} (McCully 2001). Dietary choline deficiency produces hyperhomocysteinemia because of an inhibition of transmethylation of homocysteine to methionine, causing homocystinuria by methionine synthase deficiency (McCully 2001). In 1972 a third cause of homocystinuria was discovered, a deficiency in the methyenetetrahydrofolate reductase enzyme (McCully 1983). This publication supported McCully's hypothesis that homocysteine can cause plaques by a direct

effect on the cells and tissues of the arteries regardless of the enzyme deficiency causing hyperhomocysteinemia (McCully 2001).

1.7.2 Homocysteine Theory of Atherosclerosis

During the 1960s no research showed altered protein metabolism had an effect on atherosclerosis (McCully 2001). Work by Moses Suzman, a South African cardiologist found pyridoxine (vitamin B_6) benefited patients with coronary heart disease. Through collaboration with McCully, Suzman, suggested more research on homocysteine and linking an elevation within this amino acid to the aetiology of atherosclerosis.

Ignoatowsky conducted experiments in 1908, whereby he induced atherosclerosis by feeding rabbits milk, eggs and meats. Following on from Ignoatowsky's experiments, Harry Newburgh conducted experiments in the early 1920s whereby removing all lipids and cholesterol from the meats fed to laboratory animals, so meats containing only protein were given, thus showing atherogenesis without lipid feeding. Following this experiment with rabbits, Newburgh infused amino acids intravenously in dogs, however they did not show atherosclerotic plaques in the arteries. Methionine and homocysteine were discovered in the early 1920s so the relevance of Ignoatowsky's experiments has only just become relevant. The experiments conducted by Newburgh and Ignatowsky suggested an important role in protein feeding and vitamin B_6 deficiency, with a suggested role in atherogenesis.

Experiments conducted by Klavins, showed that, homocysteine in a synthetic, man-made diet fed to rats did not produce arterial plaques (McCully 2001). The early experiments conducted by McCully involved injecting homocysteine into rabbits (McCully 1971). After three weeks of initial injections early fibrous arteriosclerotic plaques were demonstrated in coronary arteries. Rabbits in these experiments were also fed cholesterol in their diets which resulted in lipid deposition within these plaques, also the diets were deficient in vitamin B_6 which increased the severity of the plaques. Increased doses of homocysteine or methionine given to the rabbits over a 2 month period resulted in advanced plaques and the death of several rabbits from pulmonary embolism, a complication often found in children with homocystinuria. In some of the rabbits, thrombosis and embolism were prevented

by injecting vitamin B_6. Feeding rabbits a synthetic diet which contained homocysteine and methionine also produced arteriosclerotic plaques and the addition of lipids in the form of butter to the diet converted fibrous plaques to fibrolipid plaques. McCully's *in vivo* experiments were confirmed by Harker et al. (1976) where homocysteine was injected into a primate animal model. The baboons were shown to have endothelial damage, thrombosis of peripheral arteries and fibrous arteriosclerotic plaques post injection (Harker et al. 1976). The results of animal experiments *in vivo* are consistent with the hypothesis which demonstrated the atherogenic potential of homocysteine.

Early reports suggest atherosclerosis in the human population is linked to a deficiency of vitamin B_6. Food processing has been suggested to cause a significant reduction of vitamin B_6, folic acid, mineral and fibre (Schroeder 1971). McCully found that the processing of foods, milling of grains, canning, chemical additives, extraction of sugar and oils from plants were responsible for a widespread deficiency of vitamin B_6 and folic acid in populations susceptible to vascular disease (McCully and McCully 1999).

McCully's homocysteine theory of atherosclerosis in 1972 proposes that the underlying cause of vascular disease is a deficiency disease of B-vitamins as opposed to the supposed effects of dietary cholesterol and fats which is the traditional diet/heart hypothesis for atherosclerosis.

1.7.3 The Cellular Changes Associated with Arteriosclerotic Plaques Induced by Altered Homocysteine Metabolism

Cultured skin cells from patients with homocystinuria, were prepared to aid the understanding of the arteriosclerotic plaques induced in homocystinuria and experimentally induced atherogenesis (McCully 2001). Cells were found to be deficient in cystathionine synthase.

Naruszewicz (1994) observed that homocysteine thiolactone (thio-ester of homocysteine) converts LDL to a small dense form. These particles are associated with increased risk of vascular disease (Naruszewicz et al. 1994). Homocysteine

links to the apolipoprotein B (apoB) protein by a peptide bound homocysteinyl groups which causes aggregation and precipitation of the LDL particles. ApoB is an essential primary apolipoprotein of large plasma lipoproteins, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and low density proteins (LDL) and is produced in the liver (Mahley et al. 1984). The function of apolipoproteins is to transport and redistribute lipid among various tissues in the body (Kastelein et al. 2006; Mahley et al. 1984). The presence of apoB has been shown in the literature to be a high predictor of cardiovascular disease risk (Kastelein et al. 2006). The resulting homocysteine LDL aggregates are taken up by macrophages which form foam cells. The foam cells deposit in the artery wall, which leads to the deposit of cholesterol and fats within the atherosclerotic plaques, induced by the effects of homocysteine on arterial cells. These observations by McCully suggest that LDL is a carrier of homocysteine in the pathogenesis of atherosclerotic plaques. Figure 1.12 illustrates this process.

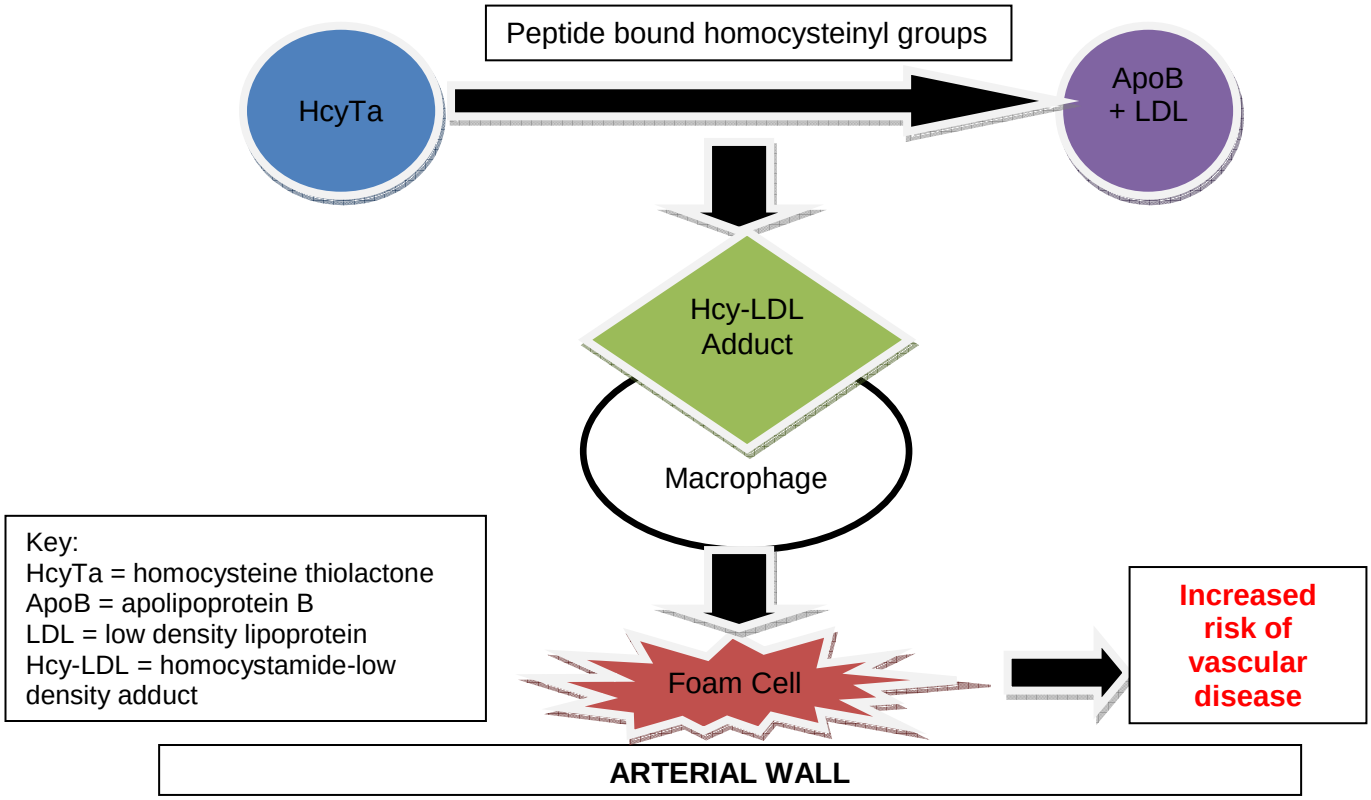

Figure 1.12: Process of formation of foam cells through the binding of homocysteine thiolactone, apoB and LDL particles. Adapted from Ferguson et al. 1999

The first clinical and epidemiological studies within human populations did not become available until 1976, when Wilcken and Wilcken (1976) published the first human study of homocysteine metabolism in coronary heart disease. A number of major studies have highlighted the potential significance of homocysteine in the pathogenesis of atherosclerosis; e.g. Physicians Health Study (1982); Framingham Health Study (1948); Nurses Health Study (1976); European Concerted Action Project on Homocysteine (1997), Hordaland Homocysteine Study in Norway (1992) and British United Provident Association Study (BUPA) (1975-1982) (McCully 2001). In 1983 McCully made the following comment: "The decline of cardiovascular disease in the United States in the past 35 years can be attributable to the addition of vitamin B_6 and folic acid to the food supply in the form of voluntary fortification of cereals and vitamin supplements". The changes in diet, particularity through the increase in alcohol consumption, which is evident in many countries, particularly the U.K. in the past decades, may through a counter productive action on homocysteine metabolism challenge the truth of McCully's above statement at the present time. This requires a clearer investigation into the exact effect homocysteine has on the cardiovascular system.

1.7.4 Previous Homocysteine and Cardiovascular Disease Studies

The first meta-analysis of the effect of homocysteine on CVD was conducted in 1995 by Boushey (Boushey et al. 1995). A study by Verhoef et al. (1998) also showed similar results whereby an increase of 1 µmol/l in fasting plasma homocysteine has been associated with an increased risk of coronary heart disease (CHD) by 10-20%. There was a strong association between vascular disease and elevated homocysteine, whereby 5 µmol/l increase was equivalent to a 20 mg/dl increase in plasma cholesterol in terms of Coronary Artery Disease (CAD) risk (Verhoef et al. 1998). Following on from this The Homocysteine Studies Collaboration meta-analysis on CAD in stroke patients concluded that a 3 µmol/l decrease in homocysteine concentration reduced the risk of CAD by 11% and stoke by 19% (The Homocysteine Studies Collaboration 2002). Clarke and Armitage 2000 state that a realistic reduction in CVD should be reached by a 10-15% reduction in the levels of homocysteine, which is of the greatest clinical and economic interest. This finding was also found by Hornberger 1998.

A number of studies have illustrated through clinical trials and experiments that diet supplementation with folate, vitamin B_6 and vitamin B_{12} have the potential to normalise plasma homocysteine levels (Appel et al. 2000; Brunner et al. 1997; Jacobsen 1998; Malinow et al. 1998; Vermeulen et al. 2000). Folate intake of at least 0.4-0.5 mg/day with or without vitamin B_6 (16.5 mg/daily) and vitamin B_{12} (0.5 mg/daily) gives optimal homocysteine concentrations (Homocysteine Trialists Collaboration 1998; Malinow et al. 1999). A definitive answer on the benefit of vitamin supplementation on reducing homocysteine levels, can only be achieved through the development and design of large scale randomised controlled trials, as demonstrated by; Clarke 1998; Clarke and Armitage 2000; Eikelboom et al. 1999; Sunder-Plassmann et al. 2000. This leads onto other studies which concluded that reducing homocysteine by 3 µmol/l through folic acid supplementation, could reduce the risk of ischemic heart disease by 16%; Deep Vein Thrombosis (DVT) by 25% and stroke by 24% (Wald et al. 2002).

Two large scale clinical trials investigating homocysteine lowering and cardiovascular disease risk were published in 2006; HOPE-2 (Lonn et al. 2006) and NORVIT (Bonaa et al. 2006). Both of these trials concluded that treatment with folic acid and vitamin B_{12} did reduce homocysteine levels in patients who had been previously diagnosed with CVD. However the reduction of homocysteine levels did not reduce the risk of ongoing CVD risk factors. These studies suggest that even though homocysteine levels have actively reduced the risk of CVD, developing ongoing related conditions were not reduced. This indicates that further research is required to investigate the exact type of B-vitamin dose and combination needed to actively reduce homocysteine concentration and the CVD risk potential. It is important to note that each study reviewed did use different doses of folic acid, vitamin B_{12} and vitamin B_6 for their interventions.

1.7.5. Current Homocysteine and Cardiovascular Disease Risk Research

In the last two years there have been more than 30 studies published in peer reviewed journals which investigate the relationship between homocysteine and cardiovascular disease (see appendix 1E). Of these studies, five were large scale trials with greater than 1000 participants and are summarised in table 1.16 (Albert et

al. 2008; Collings et al. 2008; Cui et al. 2008; Ebbing et al. 2008; Min et al. 2009). However a large proportion of these studies investigated the relationship between homocysteine levels and established cardiovascular diseases such as stroke and myocardial infarction in samples already diagnosed with these conditions.

Table 1.16: Key studies published in 2008-2009 investigating the relationship between homocysteine and cardiovascular disease.

Reference & Country	Aim	Main Findings
(Cui et al. 2008) Japan	Investigate the association of serum homocysteine and cardiovascular disease in an Asian population.	At 10 year follow-up there were 444 cardiovascular related deaths. Participants within the highest total homocysteine quartile (>15.3 µmol/l) had higher mortality, in comparison with the lower quartile (<10.5 µmol/l). High serum total homocysteine level were associated with increased mortality from ischemic stroke, coronary heart disease and total cardiovascular disease among a Japanese population.
(Albert et al. 2008) USA	Investigate intervention of vitamins B_6, B_{12} and folic acid supplementation in high risk women with and without CVD.	The intervention group's plasma homocysteine was decreased by 18.5% ($p<0.001$), by a difference of 2.27 µmol/l in comparison with placebo group. After the 7.3 year intervention, the combination pill did not decrease the risk of CVD events in high risk female, despite a significant decrease in plasma homocysteine.
(Ebbing et al. 2008) Norway	Investigate the dosing of folic acid, vitamins B_{12} and B_6 on patients with coronary artery disease and aortic valve stenosis.	Mean plasma total homocysteine concentrations were reduced by 30% after 1 year of treatment in the groups receiving folic acid and vitamin B_{12}. The trial was terminated early because of concern among participants due to preliminary results from a contemporaneous Norwegian trial, suggesting adverse reactions from the intervention. The trial did not find any effective results for the treatment of folic acid/vitamin B_{12} or vitamin B_6 on total mortality or cardiovascular events.

Reference & Country	Aim	Main Findings
(Collings et al. 2008) Finland	Investigate whether common risk variables interact with the connexin (Cx37) C1019T polymorphism causing possible interactions with early markers of atherosclerosis	Homocysteine in subjects with the TT genotype were found to be associated with higher flow mediated dilatation (FMD) values (p for interaction 0.038). Study concluded the effect of smoking and homocysteine levels on arterial endothelial functions and elasticity were modified by the allelic variation of the Cx37 gene.
(Min et al. 2009) Korea	Evaluate the usefulness of baseline homocysteine levels for predicting the risk of short or long-term cardiovascular events after successful coronary stenting.	After multi-variate logistic analysis, homocysteine levels, female sex, hypertension, multivessel diseases, American Heart Association lesion type B2/C and stent length were independently associated with peroiprocediral MI. Study concluded that preprocedural levels of homocysteine were an independent predictor for preprocedural MI and death or MI after successful coronary stenting.

The Japan Collaborative Cohort (JACC) Study for Evaluation of Cancer Risk, was a large 10 year follow-up trial (Cui et al. 2008). At the 10 year follow up stage of this study, there were 444 CVD related deaths. The subsequent data analysis showed that individuals with the highest plasma homocysteine levels (>15.3µmol/l) had greater mortality. Cui et al. (2008) concluded that high homocysteine levels were associated with increased mortality from ischemic stroke, CAD and CVD in a Japanese population. Similar results were shown in a study conducted by Bonaa et al. (2006).

A randomised double blinded placebo controlled trial conducted by Albert et al. (2008) was designed whereby folic acid, vitamin B_{12} and vitamin B_6 were given as supplements to women (N=5442) aged greater than 42 years. The 7.3 year intervention found that the folate, vitamin B_{12} and B_6 intervention did not reduce CVD risk, even though there was a 18.5% reduction in the plasma homocysteine levels ($p<0.001$). There was a 2.27 µmol/l difference in homocysteine concentrations between the intervention and placebo group. A similar trial was conducted by Ebbing et al. (2008) with a sample population who had an established diagnosis of CAD and/or aortic value stenosis. This trial was terminated before conclusion, because preliminary data showed that plasma homocysteine was reduced by 30% in the intervention group, but the intervention of supplementation with folic acid, vitamins B_{12} and B_6 did not decrease CVD risk, even in the presence of reduced homocysteine levels.

Studies have also been conducted in samples of young adults, to determine homocysteine levels. Collings et al. (2008) investigated lifestyle factors on early makers of atherosclerosis in a population of young adults (N=1440). Data was examined from the Cardiovascular Risk in Young Finns study. The study data analysis showed that participants with the MTHFR TT genotype had a higher association with flow mediated dilatation (FMD) (p for interaction 0.038).

The literature search resulted in a low number of studies which investigated cardiovascular disease risk and homocysteine levels in healthy individuals who have varying degrees of alcohol consumption and other lifestyle factors (appendix 1E). The literature search also highlighted the number of studies which investigated homocysteine levels in samples of individuals with established CVD (Freitas et al.

2008; Keles et al. 2009; Shammas et al. 2009; Vaya et al. 2008). These studies investigated the levels of homocysteine in samples of individuals where CVD is established, with the aim of investigating if homocysteine can act as a biomarker for possible CVD events. Min et al. (2009) found that homocysteine was not an independent predictor for determining risk of CVD events after coronary artery stenting. This data was presented as a poster abstract and very limited methodology was available. The published studies which are listed in appendix 1E investigated homocysteine levels in samples of patients with established CVD, did consistently show that homocysteine, either within serum or plasma, was at a higher concentration within individuals who were exhibiting signs of cardiovascular disease.

The studies which investigated actively reducing homocysteine levels in either samples, where CVD was established (Albert et al. 2008; Arnesen et al. 1995; Ghosh et al. 2009; Saposnik et al. 2009) or in healthy individual samples (Cui et al. 2008; Ebbing et al. 2008), concluded that homocysteine in either serum or plasma was significantly reduced by folic acid, vitamin B_6 and/or vitamin B_{12} intervention, however a reduction in overall risk of developing CVD was not found.

The use of folic acid at a dose of 400 µg/day, has a positive effect on the vascular wall, by improving endothelial function, lowering intracellular redox states and improving the elastic properties of large vessels *in vivo* (Antoniades et al. 2009). This was found to be effective in patients with CAD (Antoniades et al. 2009). However, increasing the dose of folic acid to 5 mg/day, did not increase the positive effects on the vascular wall and endothelium, therefore rendering this higher dose to be ineffective (Antoniades et al. 2009). This can be explained as both a high (5 mg/day) and low (400 µg/day) dose induce a similar level of 5-MTHF in the vascular endothelium, even though a higher level of 5-MTHF is induced in the plasma using the 5 mg/day higher dose, therefore a lower dose of 400 µg/day is just as effective.

Recently published literature on the effect of high dose folic acid on the myocardium within a model of ischaemia and reperfusion has shown positive effects (Antoniades et al. 2009; Moens et al. 2008). The cardioprotective effects of folic acid are potentially due to the preservation of high energy phosphates and endothelial nitric oxide synthase (eNOS) coupling, improvement of myocardial redox and prevention of mycocardial cell death (Moens et al. 2008). However this work is preliminary and

more research is needed to demonstrate the full effect of folic acid on the myocardium.

Another possible reason why folic acid supplementation does reduce homocysteine levels, but not over all CVD risk, could be the enhancement of the methylation pathway, when excess folic acid is administered (Antoniades et al. 2009). Increased methylation of arginine residues increases asymmetrical dimethylarginine (ADMA) levels, which can adversely affect clinical outcomes, by inhibiting or uncoupling eNOS (Antoniades et al. 2009). In addition to the inhibiting or uncoupling of eNOS, the altered methylation of cells can impact on gene expression; whereby hypermethylation of the promoter region of several pro-atherogenic genes, can result in the up regulation of pro-atherogenic molecules (Antoniades et al. 2009). Another important reason why CVD risk is not reduced by folic acid supplementation is through the involvement of folic acid in the synthesis of thymidine, which can lead to cell proliferation and so to worsening atherosclerosis (Antoniades et al. 2009).

The reduction of homocysteine in plasma by 25-30% can be achieved by a daily dose of 400 µg of folic acid (Antoniades et al. 2009). Homocysteine plasma levels can be further reduced by another 7% by the additional dose of 0.02-1 mg daily of vitamin B_{12} (Antoniades et al. 2009). Recent clinical trials by Bonaa et al. (2006) and Lonn et al. (2006) did not show an achievable reduction in CVD risk using the above supplementation doses, however a large scale clinical trial has yet to be conducted within a healthy patient sample, where there is no CVD present and key lifestyle factors such as alcohol consumption have been measured. The evidence in the literature that alcohol consumption, in a range of patterns has an increasing effect on plasma homocysteine levels and therefore detrimental effects on the cardiovascular system, suggests that a large scale Randomised Controlled Trial (RCT) investigating the effect of different patterns of alcohol consumption on plasma homocysteine and the subsequent risk of cardiovascular disease risk is required.

1.8 AIMS

The aim of this thesis was to investigate the effect of non-dependent and dependent patterns of alcohol consumption on cardiovascular disease (CVD) risk, though the measurement of the CVD risk biomarker, homocysteine.

The consumption of alcohol can be categorised into pre-defined patterns using the guidelines set out by the UK Department of Health (UK Department of Health 1995). These guidelines and alcohol drinking patterns led to the following objectives:

1. To recruit a sample of individuals representing a range of consumption patterns; abstainers (consume no alcohol or less than 1-2 alcoholic drinks per year), responsible drinkers (consume no more than 16-24 g (female) or 24-32 g (male) daily), sessional drinkers (consume >48 g (female) or >64 g (male) daily) and alcohol-dependent individuals (diagnosed as being dependent on alcohol by a medical professional and undergoing a medically supervised detoxification).

2. Categorise participants drinking patterns using alcohol consumption questionnaires and detailed analysis of drink diaries where appropriate.

Carbohydrate-deficient transferrin (CDT) has been shown to be effective as a biomarker of alcohol consumption when monitoring alcohol abstinence in samples of alcohol dependent individuals who are undergoing detoxification and alcohol withdrawal (Jeppsson et al. 2007). However, the use of CDT as a biomarker of alcohol consumption in a sample of individuals who consume alcohol in non-dependent patterns is less well investigated. This has led to two objectives which will:

3. Investigate the levels of serum CDT in individuals previously assigned to the alcohol dinking patterns identified above.

4. To develop a HPLC method for the detection of CDT in serum at Queen Margaret University using a published method (Helander et al. 2003).

Published literature has established homocysteine as a biomarker of cardiovascular disease (CVD) risk (Antoniades et al. 2009). Evidence has also highlighted that plasma homocysteine increases during the consumption of alcohol, as shown in alcohol-dependent patients (Bleich et al. 2000b), but this is less well investigated in

individuals who consume alcohol in a range of non-dependent patterns (Gibson et al. 2008), therefore an aim is to:

5. Investigate the effect a range of alcohol drinking patterns have on plasma homocysteine levels.

The detection of homocysteine in body fluids has been shown to be most commonly undertaken in serum or plasma. However, there have been publications which suggest that homocysteine can be detected in urine, which is a less invasive sample to collect (Thomson and Tucker 1986; Thomson and Tucker 1985). Therefore an objective is to:

6. Develop and then validate a method for the detection of homocysteine in urine using high pressure liquid chromatography (HPLC).

Circulating levels of homocysteine in plasma have been shown to increase in the presence of the $MTHFR_{(C677T)}$ polymorphism (Bennouar et al. 2007). However the prevalence of the $MTHFR_{(C677T)}$ polymorphism has not been investigated in a sample of Scottish drinkers, or been investigated in association with plasma homocysteine levels. Therefore an objective was to:

7. Determine if the presence of the $MTHFR_{(C677T)}$ polymorphism affected plasma homocysteine levels and to estimate its prevalence within a sample of Scottish drinkers, who were both dependent and non-dependent on alcohol.

The metabolism of homocysteine is dependent on two vitamin co-factors; folate and vitamin B_{12} (Mason and Choi 2005). It has been established that the consumption of alcohol lowers and depletes the circulating levels of folate and vitamin B_{12} *in vivo*. However, this finding has not been investigated in a sample of healthy individuals who consume alcohol in a non-dependent pattern (Mason and Choi 2005) Therefore, the final objective was to:

8. Investigate the levels of folate and vitamin B_{12} in the sample of individuals who consume alcohol in a range of patterns and also within the sample of alcohol dependent individuals and to investigate potential associations between the two vitamin co-factors and plasma homocysteine.

CHAPTER 2: MATERIALS AND METHODS

This materials and methods chapter relates to the following three studies: Healthy Individuals Study (HI), Alcohol Dependant Individuals (ADI) Study and the Stability Study.

2.1 Healthy Individual (HI) Study

2.1.1 Ethical Approval

Queen Margaret University (QMU) and Napier University Ethics Committee gave favourable ethical opinion for the study entitled "Varying alcohol drinking patterns in healthy individuals (HI)" (Appendix 2A, 2B and 2C) on the 29th March 2007 and 29th May 2007 respectively.

2.1.2 Research and Development (R&D) Approval

Research and development approval was not applicable for the HI study, as the recruitment for this study was not undertaken within the National Health Service (NHS) and did not involve NHS Lothian staff, patients or facilities.

2.1.3 Study Protocol

2.1.3.1 Inclusion and Exclusion Criteria

The inclusion criteria for participants in the healthy individuals (HI) studies were as follows:

Inclusion Criteria

- male or female aged 18 to 50 years
- in general good health

The inclusion criteria were justified due to the literature evidence on alcohol consumption within the age range of 18 to 50 years (Catto 2008). The 18 year minimum age for inclusion into the study was due to ethical reasons, as it would be unethical to recruit participants for this type of research who are below 18 years of age. The literature evidence suggest that sessional drinking occurs prominently in

the 18-25 year group, which includes students, graduates and young professional, identifying this as a key group to investigate in terms of drinking patterns and the subsequent comparison to the study biomarkers (Catto 2008; Gill 2002). The Alcohol Statistics 2009 report highlights that 60% of males and females aged between 16-24 years consume more than twice the recommended daily limit of alcohol (NHS Scotland 2009). Furthermore by recruiting an older age group (26-50 years), this allows for a comparison to be undertaken in terms of alcohol drinking patterns and the study biomarkers levels between the two groups. The maximum age limit for the HI study was 50 years and this is justified by evidence within the Alcohol Statistics 2009 report, which shows that alcohol consumption declines after 50 years of age within the Scottish population (NHS Scotland 2009). By recruiting only participants who have self-reported they are in good health, this minimises any other medical conditions affecting the potential correlation between alcohol consumption and the study biomarkers.

Table 2.1: HI Study Exclusion Criteria

Exclusion Criteria	Reason for Exclusion
Pregnant, trying to become pregnant or breast-feeding	Unethical to conduct this type of research on women of child bearing age due to possible risk to mother and baby (European Medicines Agency 2009; World Medical Association 2008).
Self-reported history of cardiovascular disease	Potential confounding effect of pathology on homocysteine biomarker (Antoniades et al. 2009).
Self-reported history of cancer	Potential effect of pathology on normal metabolism (Ducros et al. 2002).
Self-reported diabetes mellitus	Participants would be unable to fast for blood sampling (Ducros et al. 2002).
Self-reported gastric or duodenal ulcers	Potential confounding effect on nutritional absorption and could be damaging fasting for blood sampling (Ducros et al. 2002).
Self-reported liver or gall bladder disease	Potential effect of liver dysfunction on homocysteine and CDT biomarker levels (Antoniades et al. 2009; Das et al. 2008).
Self-reported known history of blood borne viruses	To minimise the risk to the researcher through needlestick injury (Health Protection Scotland 2010; NHS Lothian 2008).

However in the case that a blood borne viral infection was not disclosed and if a needle stick injury occurred, occupational prophylactics were available for the PhD researcher. The practices employed by the researcher while collecting data and blood samples were influenced by the safety code published by the Social Research Association, along with QMU safety guidelines (The Social Research Association 2001). This included always carrying a mobile telephone, informing the PhD researcher's supervisory team of her location, when consenting and asking participants to complete study questionnaires and further more when in a room with a participant, the PhD researcher was always positioned nearest to the door.

Participants for the HI Study were also screened for the 5,10-methylene-tetrahydrofolate reductase ($MTHFR_{(C677T)}$) polymorphism, which is a common genetic polymorphism found in more than 1% of the general population. The mutation in the gene which produces the enzyme MTHFR, prevents homocysteine being metabolised into methionine, resulting in an abnormally high circulating homocysteine concentration. If a participant was found to be positive for the $MTHFR_{(C677T)}$ polymorphism, they were informed by letter and advised to contact their general practioner (GP). They were at this point also asked to give their permission for their data to be analysed in a separate group, by signing and returning a second consent form. This was a protocol deviation from the original information shown in appendix 2G, but was approved by the QMU research ethics committee and a modified information sheet was generated as shown in appendix 2H.

The inclusion and exclusion criteria were detailed on the information sheet (Appendix 2G and 2H), which every participant was given to read before consenting to undertake this study. Once a participant had decided to take part in this study, a consent form was dated and signed by the participant and researcher (Appendix 2I).

2.1.3.2 Recruitment of Participants

Participants for the HI study were recruited from QMU and Napier University in Edinburgh. QMU has a 77% female student population, compared to Napier University, where 54% of students are female. It is therefore anticipated that recruitment from QMU would be biased towards females and formed part of the exploratory nature of the HI study. Recruitment was undertaken using the university controlled institutional e-mail system (which were vetted by information technology staff within the university then sent out to all students and/or staff on the university email system), posters and advertisements on student-centred official university intranet pages. Recruitment was directed to both staff and students from each institution and was carried out from May 2007 until March 2008. Emails were sent out to all staff and students via the QMU Moderator Email System for the duration of the 10 month recruitment period of the HI study. A permanent poster advertisement was placed on the Student and Staff Intranet pages of Napier University for the HI study recruitment period. Appendix 2F details the email and

poster advertisements employed. Potential participants were not offered any payment for taking part in the HI study, which is in line with similar studies, published in the literature that did not offer payment for taking part in research (McMahon et al. 2007). Furthermore budget restraints prevented payment for participation being offered. There is however evidence in the literature that does suggest that paying potential participants, does increase recruitment rates and retention (Gul and Ali 2010). Participants who consented to taking part in the study and had to travel to either QMU or Napier University for study appointments were offered reimbursement for their travel expenses.

2.1.3.3 Sample Size

Alcohol dependence has been shown in the literature to increase plasma homocysteine levels (Bleich et al. 2000d), however the effect of non-dependent alcohol drinking on plasma homocysteine levels has not been established within the literature. The HI study was deemed to be an exploratory study, to determine if non-dependent drinking would induce a change in plasma homocysteine levels within healthy individuals, therefore a sample size calculation was not applicable.

2.1.3.4 Recruitment Procedures

At the beginning of the study, participants who met the inclusion and exclusion criteria, were asked to attend a pre-study interview, where a consent form (Appendix 2I) and study questionnaire (Appendix 2L) were completed and signed. At this initial meeting, blood sampling appointments were also arranged. The study questionnaire was used to categorise each participant according to their age and alcohol drinking pattern. Table 2.2 details the potential categories which participants were assigned to.

Table 2.2: Participant's alcohol consumption categories

Group Number	Gender	Age Group (Years)	Alcohol Drinking Pattern
1	Male	18-25	Abstainer
2	Male	18-25	Responsible Drinker
3	Male	18-25	Sessional Drinker
4	Male	26-50	Abstainer
5	Male	26-50	Responsible Drinker
6	Male	26-50	Sessional Drinker
7	Female	18-25	Abstainer
8	Female	18-25	Responsible Drinker
9	Female	18-25	Sessional Drinker
10	Female	26-50	Abstainer
11	Female	26-50	Responsible Drinker
12	Female	26-50	Sessional Drinker

Participants were categorised as abstainers if they consumed one or less alcoholic drinks per year. Participants were categorised as a responsible drinker if they drank within the UK recommended drinking guidelines, e.g. 3-4 units/day (24-32 g/day) for a man and 2-3 units/day (16-24 g/day) for a women (UK Department of Health 1995). Participants were categorised as sessional drinkers if they consumed more than 6 units (48 g) (female) and 8 units (64 g) (male) on a single occasion (NHS Scotland 2009). In the UK one alcohol unit is defined as 8 g of pure ethanol (UK Department of Health 1995).

Each participant recruited to the HI study was given a timetable for the length of the study (table 2.3). This detailed when they would be required to keep a diet and alcohol diary and also when blood sampling appointments were due. Diet and alcohol diaries are required to be completed, to collect data on food and alcohol consumption, during the 7-day monitoring period. Participants were provided with a 7-day diet diary, which would be completed 7 days prior to blood sampling on day 8 (Appendix 2M). Diet diaries for the 3 month and 6 month time-points would be mailed to participants near to their scheduled blood sampling appointment for the later study time-points.

Table 2.3: HI Study Timetable given to Participants

Timetable	What is required of participant
Questionnaire and Introduction. Meeting at a place and time most convenient to the participant.	You will be asked to complete a questionnaire and will be given a urine sample container and a 7-day alcohol and diet diary. Female participants will be asked to return the questionnaire in a pre-paid envelope. The diet and alcohol diary should be completed for the seven days prior to the blood sampling appointment on day eight,
Day 1 (Baseline) at QMU	You will be asked to bring to the meeting a waking urine sample, and the completed alcohol and diet diary. Fasting* blood sample will be taken, by the PhD researcher. You will be given another 7-day diet and alcohol diary, to complete for the seven days prior to blood sampling on day eight. An appointment for 3 months time will be made to return to QMU for blood and urine sampling.
Day 2 (Three months from date of baseline sampling) at QMU	You will be asked to bring to the meeting a waking urine sample, and the completed alcohol and diet diary. Fasting* blood sample will be taken, by the PhD researcher. You will be given another 7-day diet and alcohol diary to complete for the seven days prior to blood sampling on day eight. An appointment for 3 months time will be made to return to QMU for blood and urine sampling.
Day 3 (Six Months from date of baseline sampling) at QMU	You will be asked to bring to the meeting a waking urine sample, and the completed alcohol and diet diary. A final fasting* blood sample will be taken, by the PhD researcher.

*You should not eat 12 hours before the time of your blood sampling appointment. For example if your appointment is at 8 am you should eat nothing after 8 pm the previous night. Once the blood sample has been taken you will be offered a soft drink and biscuits before you leave.

2.1.3.5 Study Questionnaire

The HI study questionnaire is shown in Appendix 2L. The study questionnaire was piloted among a selected group of staff and students from QMU (N=10). The group was asked for feedback on the questionnaire, regarding ease of completion and understanding of each question. After feedback was complete from the pilot group, no changes were required. For this study the questionnaire was designed to gain information relating to participant's self-reported drinking frequency, pattern and volume consumed.

The study questionnaire is a three page document with nine questions, designed to be completed in 10 minutes. The first section of the questionnaire collected demographic information, including age, gender and occupation (i.e. student or employee). The questions in section two were designed to collect information on the amount of alcohol consumed, either in units or grams, which could then be compared quantitatively to the study biomarkers. The questions on alcohol consumption were designed in reflection to other alcohol consumption questionnaires used by the UK Government (Office of National Statistics) and other health screening bodies and have been published in the literature (Catto 2008; Goddard 2001). Validated questionnaires which are used as screening tools, including AUDIT and CAGE were not used, as these questionnaires screen for alcohol dependency and problem drinking and do not give information on units or grams of alcohol consumed. Retrospective questionnaire data could then be compared to data from diet diaries which were completed prospectively.

Female participants only were asked to complete the last page of the questionnaire, which related to the participant's menstrual cycle and contraceptive use. To respect female's privacy, the option to complete this section, of the questionnaire in private, was given. This information was included in the questionnaire as the literature suggests a link between menopausal status and homocysteine levels (Russo et al. 2008).

2.1.3.6 Biological Sample Collection

2.1.3.6.1 Venepuncture and Blood Sample Processing

A fasting blood sample was taken from each participant at three pre-arranged time points. Participants were asked to fast for 12 hours prior to their blood sampling appointment.

Each participant was asked to sit in an upright position with their chosen arm outstretched and supported by a pillow. A 21G (Greiner Bio-One, Brunel Way, Stroudwater Business Park, Stonehouse, GL10 3SX) hypodermic needle was inserted into a suitable vein in the anti-cubital fossa. Blood (9 ml) was withdrawn into an EDTA vacuette (Greiner Bio-One) and 16 ml of blood withdrawn into two serum clot activator vacuettes (Greiner Bio-One).

Serum clot activator vacuettes were placed in a refrigerator (to allow whole blood to clot), for 30 minutes. Following this, the serum clot activator vacuettes were centrifuged (Jouan BR3.11, Jouan Ltd, 10 rue Duguay Trouin, 44807 Saint-Herblain) at 3000 rpm (1000 *g*) for 10 minutes at room temperature, to produce serum. Serum was aliquoted into 1.5 ml eppendorf tubes (Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG) and stored at -80 ºC, awaiting CDT analysis by the HPLC method outlined by Helander et al. (2003) and combined Folate/Vitamin B_{12} assay at the Royal Infirmary, Edinburgh.

Anticoagulated blood (5 µl) was removed from the EDTA vacuette prior to centrifugation for DNA extraction (Rudbeck and Dissing 1998). EDTA vacuettes (Greiner Bio-One) containing anti-coagulated blood were centrifuged (Jouan) at 3000 rpm (1000 *g*) for 5 minutes at 4 ºC to produce plasma. Plasma was aliquoted into 1.5 ml eppendorf tubes (Fisher Scientific) and stored at -80 ºC, awaiting homocysteine analysis by HPLC (Cummins 2005; Houze et al. 2001).

Serum and plasma samples from HI study participants were stored at -80 ºC. Urine samples from HI study participants were stored at -20 ºC. All biological samples collected from HI study participants were stored at QMU.

2.1.3.6.2 Urine Collection

Participants were asked to produce a waking urine sample on the morning of their blood sampling appointment. Urine was collected in 30 ml universal containers (Fisher Scientific) and stored at -20 ºC, awaiting homocysteine analysis by HPLC

2.1.3.6.3 Diet Diary Analysis

Each participant in the HI study was asked to record their diet and alcohol consumption for 7 days leading up to blood sampling on day 8. Participants were supplied with a diet and alcohol diary which were adapted from Gregory et al. 1995 (appendix 2M). In total, participants each recorded three diet and alcohol diaries over the course of the 6 month HI study, at the baseline, 3-month and 6-month time-points.

Participants completed 7 day prospective diet diaries which were analysed using WinDiets 2005 (WinDiets Research Version, Univation Ltd; c/o The Robert Gordon University; CREDO; Schoolhill; Aberdeen; AB10 1FR). Diet diaries were used to analyse folate and vitamin B_{12} intake (section 2.4.6) as well as alcohol consumption (section 2.3). For the diet diary analysis a selection of random participants (N=7) were chosen to have their complete diet diaries analysed using WinDiets and the results of this analysis compared with the biological folate and vitamin B_{12} levels. This enabled a comparison of the data and permit investigation of the level of agreement between folate and vitamin B_{12} intake compared with the levels found *in vivo.*

2.2 Alcohol Dependent Individual (ADI) Study

2.2.1 Ethical Approval

NHS Research Ethics Committee (LREC 02) gave favourable ethical opinion for the study entitled "CVD and alcohol biomarker measurements in alcohol-dependant individuals (ADI)" (Appendix 2D) on the 20th July 2007.

2.2.2 Research and Development (R&D) Approval

The Lothian NHS Trust granted research and development approval for this study on 27th September 2007 (Appendix 2E).

2.2.3 Study Protocol

2.2.3.1 Inclusion and Exclusion Criteria

The Inclusion criteria for the ADI study were as follows:

- Male or female aged 18-55 years
- Patients undergoing alcohol detoxification as an out or in-patient of the Alcohol Problems Service (APS) in Edinburgh.
- Prescribed Chlordiazepoxide, which is a benzodiazepine, to control and aid withdrawal symptoms.

The protocol for the ADI study was based on a previously published study by Bleich et al. (2000) and the inclusion criteria reflected the Bleich protocol. The maximum age limit for the ADI study was increased after advice from the collaborating clinician at the APS, due to a higher prevalence of individuals who are admitted to the in-patient clinic, being over the age of 50 years.

The exclusion criteria for the ADI study are shown in table 2.4. Patients were screened by the admissions doctor, for the exclusion criteria before being offered the study information sheet. Patients in the ADI study did not self-report to the researcher any medical conditions outlined in table 2.4. The prescribing of the two B-vitamin supplements, Pabrinex and Thiamine were also recorded (dose per day). Thiamine (vitamin B_1) is routinely prescribed as an oral supplement to alcohol-

dependent individuals, whereas Pabrinex (vitamins B_1, B_2, B_6, B_3, (nicotinamide) and vitamin C) is given as an intra muscular injection when patients exhibit severe malnutrition symptoms; including diarrhoea, vomiting or have unsteady gait, peripheral neuropathy, or any signs of brain damage. The prescribing of Thiamine and Pabrinex is by the clinical judgement of the admission doctors and not prescribed as the result of blood test results. Thiamine and Pabrinex preparations do not not contain vitamin B_{12} or folic acid.

The safety of the researcher during the data collection of the ADI study is explained in section 2.1.3.1.

Table 2.4: ADI Study Participant Inclusion and Exclusion criteria

Exclusion Criteria	Reason for Exclusion
Trying to become pregnant, pregnant or breast-feeding	Unethical to conduct this type of research on women of child bearing age due to possible risk to mother and baby (European Medicines Agency 2009; World Medical Association 2008).
History of alcohol withdrawal seizures; being prescribed anticonvulsants and/or history of alcohol-induced cerebral disease or lesions	Literature suggests withdrawal seizures can affect homocysteine concentrations (Bleich et al. 2000a).
Prescribed methotrexate	Inhibits the action of folic acid. Risk factor for hyperhomocysteinaemia (Bleich et al. 2001).
Prescribed folate supplements	Folate supplementation would affect folate assay results (Bleich et al. 2000d)
History of cardiovascular disease	Potential confounding effect on homocysteine metabolism (Ducros et al. 2002).
History of cancer	Potential effect on normal metabolism (Ducros et al. 2002)
Diabetes mellitus	Unethical to ask patient to provide a fasting blood sample (World Medical Association 2008).
Gastric or duodenal ulcers	Potential effect on nutritional absorption and could be damaging fasting for blood sampling (Ducros et al. 2002).
History of established liver cirrhosis from medical history	Change in liver metabolism due to disease process (Bleich et al. 2000d).
Known history of blood-borne viruses	To minimise the risk to the researcher through needle stick injury (Health Protection Scotland 2010; NHS Lothian 2008).

The safety of the researcher while undertaking the ADI study followed the same guidelines as stated in section 2.1.3.1. Patients within the ADI Study were also screened for the $MTHFR_{(C677T)}$ polymorphism. As this screening was the same for HI study participants, the same conditions applied, as detailed in section 2.1.3.1.

The inclusion and exclusion criteria were detailed on the information sheet (Appendix 2J), which every participant was given to read before consenting to undertaking this study. Once a participant had decided to take part in this study, a consent form was dated and signed by the participant and PhD researcher (Appendix 2K).

2.2.3.2 Recruitment of Participants

Participants for the ADI study were recruited as either out-patients undergoing detoxification from the Alcohol Problems Service (APS) or from in-patient admissions to the Ritson Clinic, which is an alcohol detoxification ward, within the Royal Edinburgh Hospital. Recruitment was undertaken by admission doctors from the alcohol problems service (APS), who used their clinical judgements to approach patients who they deemed clinically suitable for the study and met the inclusion and exclusion criteria. The ADI study was overseen and supported by Dr Jonathan Chick, consultant psychiatrist at the APS. Recruitment was carried out from October 2007 until December 2008.

2.2.3.3 Sample Size

The ADI study design was reviewed by QMU statistician, Mr Robert Rush. The required sample size for the ADI study was calculated to be 31 which reflects the large effect size of 0.55 between days 1 and 3 in homocysteine levels, and the proposed paired T-test analysis with an attrition of approximately 10%. A previous study by Bleich et al. (2000) has shown an effect size of this magnitude is achievable with an effect size of approximately 1.0 from day 0 to day 3.

2.2.3.4 Recruitment Procedures

At the beginning of the study, participants who met the inclusion and exclusion criteria as determined by the APS admissions doctor, were asked if they wished to undertake the study. If verbal consent was given by the patient, the researcher was then contacted and an appointment for the next morning was organised. In the event that verbal consent was given, the patient was advised by the admissions doctor to fast for the blood sample, which would be taken the following day. On Day 1 of the study the PhD researcher met with the patient, where the consent form (Appendix 2K) and study questionnaire (Appendix 2N) were completed, day 1 fasting blood samples were taken and a waking urine sample was given. The study questionnaire is a three page document, designed to be completed in 10 minutes. The participant was then informed of the study timetable, which is shown below in table 2.5.

Table 2.5: ADI Study Timetable for Participants:

Timetable	What is required of participant
Questionnaire at the Ritson Clinic or Alcohol Problems Clinic (Out-patients). Day 1 (Baseline) at Ritson Clinic or Alcohol Problems Clinic (Out-patients).	You will be asked to complete a questionnaire. Female participants will be asked to complete a section of the questionnaire in private. Fasting* blood sample will be taken, by PhD researcher. Waking urine sample.
Day 3 at Ritson Clinic or Alcohol Problems Clinic (Out-patients).	Fasting* blood sample will be taken, by PhD researcher. Waking urine sample.

* You should not eat 12 hours prior to your blood sampling appointment.

Female participants were asked to complete the last page of the questionnaire in private as this related to the participants menstrual cycle and contraceptive use. To respect female's privacy and confidentiality, the option to complete this section, of the questionnaire in private, was given.

2.2.3.5 ADI Study Questionnaire

The ADI study questionnaire is shown in appendix 2N. The study questionnaire was piloted among the clinical staff of the Ritson Clinic, which included the nursing staff, admissions doctors and Dr Chick (consultant). The medical staff were asked to give their clinical opinion of the functionality and ease of completeness and understanding of each question and the questionnaire as a whole. After feedback from piloting the questionnaire to clinic medical staff, no changes were made to the questionnaire.

The study questionnaire is a three page document with twelve questions, designed to be completed in 10 minutes. The first section of the questionnaire collected demographic information, including age, gender and smoking status. The following section asks questions' relating to the length of time the participant has been dependent on alcohol, followed by the type, frequency and grams of alcohol consumed on a typical drinking day. The questions in the study questionnaire were used to gauge the amount of alcohol patients consumed, prior to entering alcohol detoxification treatment and were based on similar questions asked within the study by Bleich et al. (2000). Validated questionnaires including AUDIT and CAGE were not used as the patient sample were already clinically diagnosed as being alcohol-dependent. The retrospective questionnaire was used at baseline for detailing alcohol consumption of the ADI patients who agreed to participate in the study. Patients were asked to detail their alcohol consumption pre-admission to the detoxification ward (volume of alcohol consumed of a typical day), frequency of consumption, type of alcohol consumed and the date of their last alcohol drink.

Female participants were also asked to give details relating to their menstrual cycle, contraceptive use and menopausal status as described in section 2.1.3.5.

2.2.3.6 Biological Sample Collection

2.2.3.6.1 Venepuncture and Blood Sample Processing

A fasting blood sample was taken from each participant on day 1 and day 3 (see table 2.5). Participants were asked to fast for 12 hours prior to their blood sampling appointment.

Venepuncture and blood sample processing was conducted as described in section 2.1.3.6.1. Due to NHS standardised venepuncture practises, the blood collection system known as Monovette was used (Sarstedt Ltd, 68 Boston road, Beaumont Leys, Leicester, LE4 1AW).

The medication which study participants were prescribed was recorded from admission (day 0) to completion of study (day 3). The only medications recorded were chlordiazepoxide, thiamine and Pabrinex.

Serum, plasma and urine for the ADI study was stored at the Alcohol Problems Service clinic at 35 Morningside park, Edinburgh. Serum, plasma and urine were stored at -20 ºC. According to the review by Ducros et al. (2002) serum, plasma and urine can be stored at -20 ºC or -80 ºC, without affecting the stability of homocysteine.

2.2.3.6.2 Urine Collection

Participants were asked to produce a waking urine sample on the morning of their blood sampling appointment. Urine samples were stored as described in section 2.1.3.6.2.

2.3 Alcohol Consumption Analysis

This section describes the methods for analysing the alcohol diaries which participants were asked to complete for the HI study. The questionnaires for both the HI and ADI studies, used the Alcohol Manufacturers (AM) method to analyse the grams alcohol consumed, stated in questions six and nine respectively.

Each participant kept an alcohol diary for seven days prior to their blood sampling appointment, on day eight. In the diet diary, each participant recorded all alcoholic beverages they consumed, including where possible if the beverage was self-poured, the volume, the percentage Alcohol by Volume (%ABV) and the alcohol brand name.

Participant's alcohol consumption was calculated using three different methods:

1. According to the method adapted by the Office of National Statistics (ONS) questionnaire (Goddard 2001). ONS questionnaires quantify alcohol consumption in terms of unit content per drink, for example: one alcohol unit is defined as half a pint of beer/lager/cider, small glass of wine or one measure of spirit (Goddard 2001).

2. WinDiets 2005 dietary software (WinDiets Research Version, Univation Ltd). Analysis using WinDiets software provides a data output showing the grams of alcohol consumed per day by each participant. The software provides a list of alcohol beverages, which the researcher selects, from information provided by the participants. This is shown in figure 2.1. WinDiets software then calculates the grams of alcohol consumed daily and over the duration of the diet diary period. WinDiets however does not provide alcohol information in relation to %ABV of alcohol consumed, but more simply as a type of alcohol e.g. cider, beer, lager. Also it does not take into account of the wide variety of drinks with a range of %ABV regularly consumed in the UK, e.g. 11% ABV white wine.

3. Using the information recorded by each participant within their diet diary this was then analysed using Alcohol Manufacturers (AM) information. The third method, was created by the researcher, where all common branded drinks

commercially available in the UK were listed and the grams of alcohol were calculated per volume of alcoholic beverage that is available or served in licensed premises; for example, pint, half pint, large measure or small measure. This method was recorded in the form of a spreadsheet (Appendix 2O). The most common brands of alcoholic beverage were selected from www.drinkaware.co.uk and major supermarkets. Any alcoholic beverage that a participant consumed that was not already recorded in the spreadsheet was added. The following formula was used to calculate the grams of alcohol in each volume that was consumed:

No of UK Units in Alcoholic Drink = %ABV x Volume of drink (ml)/1000

Grams of Ethanol in Alcoholic Beverage = No of U.K. Units X 8

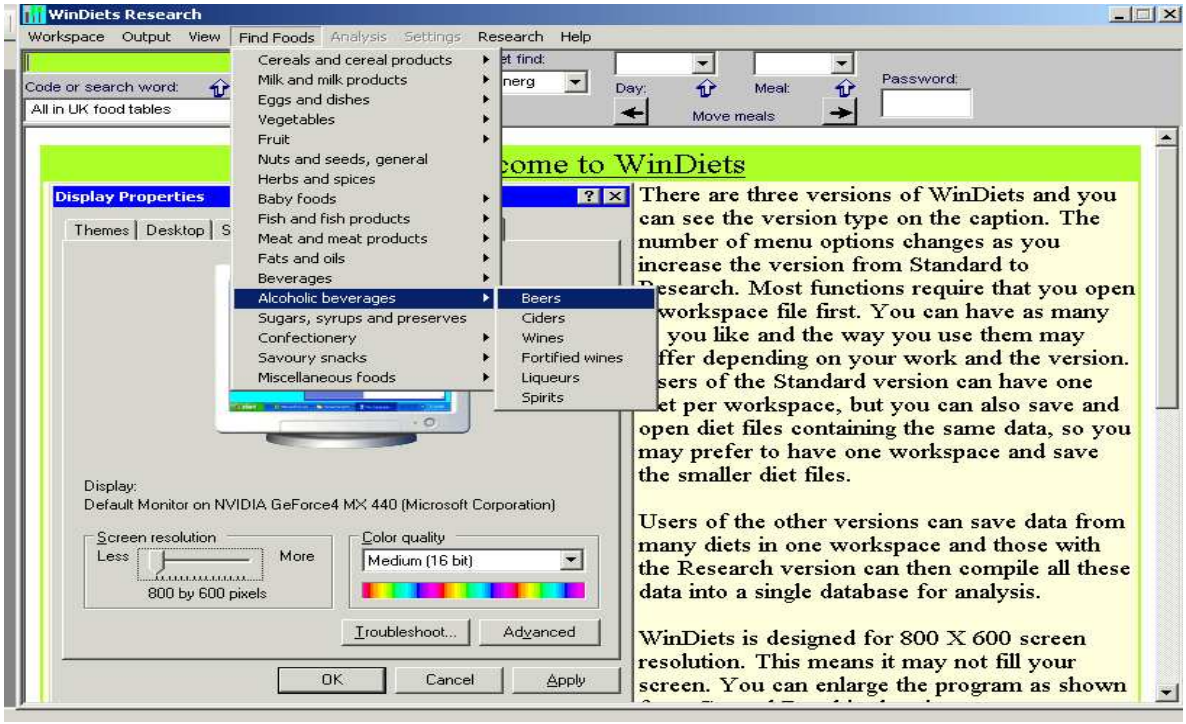

Figure 2.1: Alcoholic beverages provided by WinDiets 2005

Participant's alcohol diaries and questionnaires were analysed according to the standard measures listed in tables 2.6 and 2.7. When a participant recorded a brand name of alcohol, that specific brand was used for the analysis. In the cases where a participant did not record the brand name of the alcoholic beverage they consumed, a standard % ABV measurement was used from table 2.7.

Table 2.6: Standard Measures

Standard Alcoholic Beverage Measure	Volume (ml)
Small Measure	25
Large Measure	50
Small Wine Glass	175
Large Wine Glass	250
Pint	568
Half Pint	284
Can	440
Bottle (beer/lager)	330
Bottle (wine)	750

Table 2.7: Standard %ABV

Alcoholic Beverage	%ABV
Wine	12
Champagne and Sparkling Wine	12
Beer	4
Cider/Perry	5
Lager	5
Light Spirit	37.5
Dark Spirit	40
Fortified Wine	18
Designer Drink or Ready to Drink (RTD) e.g. Smirnoff Ice or WKD	5

Each participant's number of drinking days were recorded from their diet diary and alcohol consumption during drinking days was calculated using each of the three analytical methods. In addition the number of drinking days and alcohol free days within the period of the alcohol diary were recorded.

2.4 Laboratory Analysis

This section describes the laboratory analytical techniques which were undertaken for the HI and ADI studies.

2.4.1 Method Development for the Detection of Carbohydrate-Deficient Transferrin (CDT) in Serum

Method development was carried out at QMU and also at the Edinburgh Protein Production Facility (EPPF) within the University of Edinburgh. The Edinburgh Protein Production Facility is supported by The Wellcome Trust and the Scottish University Life Sciences Alliance.

2.4.1.1 HPLC Apparatus

At QMU the chromatography system consisted of a Waters 1525 Binary HPLC (High Pressure Liquid Chromatography) Pump (Waters) and a Waters 717 plus Autosampler (Waters). The CDT metabolites were detected by a Waters 2465 Ultraviolet (UV) Detector (Waters) at a wavelength of 470 nm. The UV detector was connected to a Dell computer which controlled the HPLC data acquisition and integration using Breeze data management software (Waters). Separation and quantification of CDT was performed on a Clarity Wax column 150 x 4.6 mm (Phenomenex).

At the EPPF, the chromatography system consisted of an ÅKTA Purifier 10 HPLC (High Pressure Liquid Chromatography) pump with a buffer preparation and an eight position buffer value (GE Healthcare, Pollards Wood, Nightingales Lane, Chalfont St, Giles, Bucks, HP8 4SP). The CDT metabolites were detected by a UV900 Ultraviolet (UV) Detector (GE Healthcare) at a wavelength of 470 nm. The UV detector was connected to a Dell computer which controlled the HPLC data acquisition and integration using Unicorn data management software (GE Healthcare). Separation and quantification of CDT was performed on a Clarity Wax column 150 x 4.6 mm (Phenomenex).

2.4.1.2 Preparation of Samples

For method development pooled serum samples were used, which were collected for the quality control stability study. The pooled serum samples used for the CDT method development were excess samples collected and utilised for the method development.

Samples were thawed overnight at 4 ºC and centrifuged (Microfuge 22 R Centrifuge, Beckman Coulter) at 6000 rpm (3500 *g*) for 5 minutes at 4 ºC before analysis. Serum (100 µl) was mixed with 20 µl of Ferric Nitrilotriacetic acid (FeNTA) (Sigma) solution. The FeNTA solution was prepared using 275 mg Nitrilotriacetic (NTA) (Sigma) and 270 mg Ferric Chloride ($FeCl_3$) (Sigma) which were dissolved in 90 ml of distilled water and pH adjusted to 7.0 with 1 mol/l Sodium Hydroxide (NaOH) (Sigma). Distilled water was added to give a final volume of 100 ml. The FeNTA solution was stored at 4 ºC. Dextran Sulphate-Calcium Chloride ($CaCl_2$) (both Sigma) solution was prepared using equal volumes of 20 g/l dextran sulphate and 1.0 mol/l $CaCl_2$, and was stored at 4 ºC. To the sample 20 µl of Dextran Sulphate-$CaCl_2$ was added and after gentle mixing, samples were left in the cold (4 ºC) for 30-60 minutes. The use of Dextran Sulphate-$CaCl_2$ as a lipoprotein precipitating agent has been reported in the literature and has an efficiency of between 90-95% at removing lipoproteins from serum (Walton and Scott 1964). Samples were then centrifuged at 6000 rpm (3500 *g*) at 5 ºC for 5 minutes. The prepared serum sample was centrifuged at 5 ºC to keep the temperature constant after addition of Dextran Sulphate-$CaCl_2$ solution. Supernatant (100 µl) was diluted with 400 µl of distilled water and transferred to a glass HPLC vial for HPLC at QMU. The diluted serum sample was injected manually onto the HPLC instrument at the EPPF, using a 1 ml syringe. The serum sample was diluted with distilled water, so as to keep the sample within the reference range of transferrin, which has been established as 0.5-5.0 g/l in serum (Helander et al. 2003).

During the method development of this assay, the sample preparation was altered to increase the iron saturation and lipid precipitation within the serum sample. This section of the method development was a result of the first HPLC sample runs, which showed that the transferrin glycoforms which were eluting from the column were not fully iron saturated. The sample preparation was altered to increase the

volume of FeNTA solution from 20 µl to 40 µl and the volume of the lipid precipitation solution was also doubled to 40 µl. The rest of the sample preparation remained the same.

It is important to state that standards were not used as they were not commercially available. Therefore the retention time was based on the times published in the paper by Helander et al. (2003). The lack of standards for CDT HPLC analysis has been reported in the literature and clearly stated in the method paper by Helander et al. (2003), which was the basis for this method development. Recent publications have stated that a standard of disialotransferrin, will soon become commercially available (Oberrauch et al. 2008).

2.4.1.2.1 QMU Mobile Phase Gradient

The mobile phase was developed according to the method of Helander et al. (2003). The Water HPLC system which was used for this method at QMU consisted of two pumps and two buffer lines, which resulted in the mobile phase gradient being altered from the original method. The aqueous mobile phase consisted of two buffer solutions: 10 mmol/l Bis-Tris adjusted to pH 6.2 with 2 mol/l HCl (buffer A) and 2.0 mol/l NaCl (buffer D) adjusted to pH 6.2 with 2mol/l HCl. The mobile phase was degassed in a bath type sonicator (Decon). The mobile phase ran as a gradient according to table 2.8, as developed from Helander et al. (2003).

Table 2.8: Mobile Phase Gradient Profile for CDT HPLC Method adapted from Helander et al (2003).

Time (Min.Sec)	Buffer A %	Buffer D %
0.00	100	0
1.00	100	0
30.00	95	5
30.01	0	100
35.00	0	100
35.50	100	0
37.00	100	0

2.4.1.2.2 EPPF Mobile Phase Gradient

The HPLC (GE Healthcare) instrument at the EPPF consisted of two pumps and multiple buffer inlet lines, which allowed for the gradient to be set up as per the method by Helander et al. (2003). The aqueous mobile phase consisted of four buffer solutions: 10 mmol/l Bis-Tris adjusted to pH 7.0 with 2 mol/l HCl (buffer A); 10 mmol/l Bis-Tris and 0.5 mol/l NaCl, pH adjusted to 6.2 (buffer B); 10 mmol/l Bis-Tris with pH 6.2 (buffer C) and 2.0 mol/l NaCl (buffer D). Each mobile phase buffer were filtered through 0.2 µm filters (Scientific Laboratory Supplied Ltd, Wilford Industrial Estate, Nottingham, NG11 7EP) and degassed under vacuum. A mobile phase gradient according to the published method was programmed into the HPLC instrument at the EPPF, shown in table 2.9. As part of the method development of this assay a second mobile gradient profile was developed and adapted from the published method and this shown in table 2.10.

Table 2.9: Mobile phase gradient profile for CDT HPLC method at EPPF (Helander et al. 2003).

Time (Min.Sec)	**Buffer A %**	**Buffer B %**	**Buffer C %**	**Buffer D %**
0.00	100	0	0	0
1.00	100	0	0	0
1.01	0	0	100	0
30.00	0	20	80	0
30.01	0	0	0	100
35.00	0	0	0	100
35.50	100	0	0	0
37.00	100	0	0	0

Table 2.10: Developed and adapted mobile phase gradient profile for CDT HPLC method at EPPF (Helander et al. 2003).

Time (Min.Sec	Buffer A %	Buffer B %	Buffer C %	Buffer D %
0.00	100	0	0	0
1.00	100	0	0	0
1.01	0	0	100	0
30.00	0	100	0	0
30.01	0	0	0	100
35.00	0	0	0	100
35.50	100	0	0	0
37.00	100	0	0	0

2.4.1.3 Running Conditions

At both QMU and the Edinburgh Protein Production Facility (EPPF), HPLC was performed at room temperature (21 ºC). Flow rate was 1.0 ml per minute. Under these conditions and according to the published method by Helander et al. (2003), the peaks representing the CDT glycoforms eluted approximately at the following times: disialotransferrin eluted at 16 minutes; trisialotransferrin eluted at 18 minutes; tetrasialotransferrin eluted at 21.5 minutes and hexasialotransferrin eluted at 26.5 minutes, with a total run time of 40 minutes.

2.4.1.4 Fractionation

A benefit of the GE Healthcare HPLC within the EPPF, was the ability to collect fractions over the period of the HPLC run. During the method development of this assay, the mobile phase gradient which is shown in table 2.10, produced the most evident peak of the transferrin protein, however this was a single peak, as opposed to the anticipated, four peaks, of each of the transferrin glycoforms. The HPLC method programme was altered to include the collection of fractions during the run. The volume of fraction which was collected was 250 µl. To identify the exact content of the peak which eluted at 15 minutes, fractions were collected across this

peak, as shown in figure 2.2. Fractions which were collected were 1E8, 1E9, 1E10, 1E11, 1E12, 1F1, 1F2, 1F3. The fractions collected were then subsequently analysed using protein gel electrophoresis and mass spectrometry.

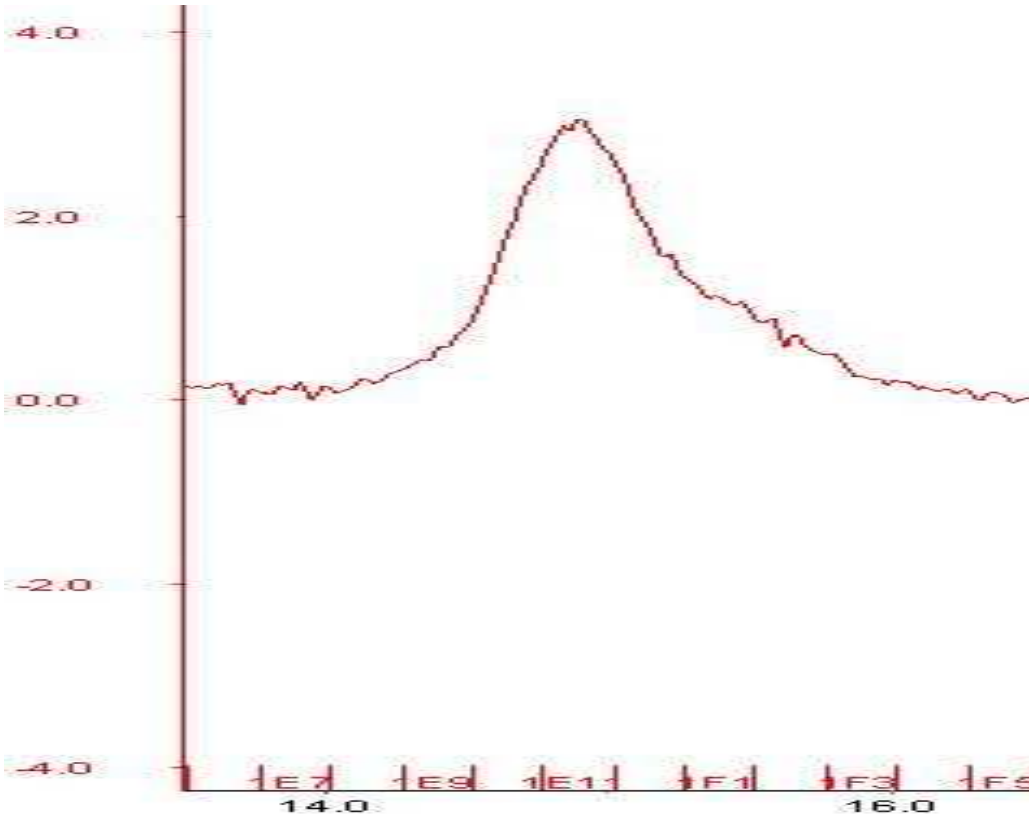

Figure 2.2: Fractions collected using HPLC with UV detection (X-axis: Retention time (minutes); Y-Axis: Wavelength (nm)

2.4.1.5 Protein Gel Electrophoresis

The approximate molecular weight of each of the fractions obtained in 2.4.1.4 were analysed using pre-prepared 4-20% Polyacrylamide LongLife gels (NuSep Ltd, 22 Rosborough Road, Frenchs Forest, NSW, 2086, Australia) which identified the approximate molecular weight of each fraction collected by HPLC with UV detection. The LongLife gels were placed within a Mini-Protean Tetra Cell electrophoresis tank (Bio-Rad) and filled with 1X Tris-Hepes SDS running buffer (NuSep). Each well was flushed with running buffer before samples were loaded to ensure the sample completely filled each well. TruSep sample loading buffer (NuSep) (10 µl) was

added to each 10 µl fraction sample and mixed well to ensure homogeneity. Within each well 20 µl of sample was aliquoted. For each gel a 250 kDa Precision Plus Protein Standard (Bio-Rad) marker was used to identify band size. Gel electrophoresis was carried out at 150 volts for 40 minutes. Once electrophoresis was complete the gels were removed from the plastic casing and washed every 5 minutes over a 15 minute period, in distilled water. During the washing process the gels were placed in plastic sealed containers containing distilled water and placed on a Gyro-Rocker SSL3 bench top shaker (Stuart Scientific), to facilitate the washing process.

Polyacrylamide gels were stained with Instant Blue, which is a Coomassie based staining solution for protein gels (Generon Ltd, Rawcliffe House, Howarth road, Maidenhead, Berkshire SL6 1AP). Gels were subjected to staining for 10 minutes then rinsed with distilled water and returned to the Gyro-Rocker SSL3 bench top shaker (Stuart Scientific) for 1 hour before photography; this was to ensure all stain was removed and banding allowed to develop.

Polyacrylamide gels were photographed under white light using a Gel Doc XR Gel Scanner Molecular Imager (Bio-Rad). Quantity One V4.6.5 software (Bio-Rad) was used to identify protein bands.

2.4.1.6 Desalting and Concentration of Fractions

Mass spectrometry is a sensitive technique used to detect the presence of a specific protein within biological samples. The content of salt within a serum sample can cause interference within the mass spectrometry analysis; the de-salting of serum samples reduces the interference thus providing a more detailed mass spectrometry identification. Also, by concentrating the fractions, by removing excess water and salt the sample therefore becomes more potent in terms of protein concentration, aiding the mass spectrometry analysis.

Desalting of the fractions involved subjecting each sample to a run through a desalting column, to remove all salt content from the fraction sample. As this

method involved a high sodium chloride buffer, this was an important step to achieve a more detailed and clearer mass spectrometry result.

The column used was a HiTrap Desalting column (GE Healthcare). The mobile phase used was 20 mM ammonium acetate (Fisher Scientific) at pH 6.8. Ammonium acetate was used as the mobile phase buffer during the desalting process, as the literature suggests that the transferrin protein is stable within this buffer (Estela de Castillo Busto et al. 2005). The fraction sample (250 µl) was manually injected directly into the HPLC system using a 1 ml syringe (BD Plastipak). Flow rate was 5 ml per minute. The HiTrap column, removed all salt ions from the sample, and the protein elutes from the column separately due to its larger molecular weight. The protein fraction, which is now free of salt, was collected as fractions into a 96-well Masterblock plate (Grenier Bio-One) in volumes of 250 µl.

To increase the concentration of the fractions which were free of salt ions, the fractions were subjected to a concentrating procedure, where all excess ammonium acetate buffer was removed, thus making the sample a purer protein sample. Before use, the VivaSpin 500 30 KDa Molecular Weight Concentrating eppendorfs (Scientific Laboratory Supplies Ltd) were washed with ammonium acetate buffer, by aliquoting 200 µl of ammonium acetate buffer into each tube and centrifuging at 6000 rpm (2000 *g*) for 5 minutes at 4 ℃. The ammonium acetate buffer was then removed from the collection section of the eppendorf and discarded. The 250 µl fraction sample was aliquoted into a VivaSpin 500 30 KDa Molecular Weight Concentrating eppendorfs and centrifuged at 2000 rpm (500 *g*) for initially 3 minutes at 4 ℃. Samples were centrifuged until they reached the 25 µl level marker on the tube. This required close monitoring of the samples during the centrifugation period, which is why a definitive centrifugation time is not given.

2.4.1.7 Mass Spectrometry

To identify the protein(s) contained within the peak that eluted from the HPLC column at 15 minutes, the fractions collected over that peak, were subjected to mass spectroscopy (Matrix-Assisted Laser Desorption/Ionization-Time of Flight

(MALDI-TOF)). The MALDI-TOF mass spectroscopy was conducted at the University of Edinburgh.

Published literature indicate that the molecular weight of the CDT glycoforms are: Disialotransferrin 77365 Da; Trisialotransferrin 79281 Da; Tetrasialotransferrin 79573; Pentasialotransferrin 80232/80379 Da (Oberrauch et al. 2008).

Fractions were prepared for MALDI-TOF mass spectroscopy as follows: 0.5 µl of fraction was spotted onto the MALDI metal 96 well plate, followed by another spot of 0.5 µl sinapinic acid (Sigma). The function of sinapinic acid was to immobilise the sample before being subjected MALDI-TOF mass spectroscopy (Estela de Castillo Busto et al. 2005). The volume in each well was 1 µl. The sample wells were allowed to dry for 30 minutes. The samples were analysed using a PerSeptive Biosystems Vovager-DE STR BioSpectrometry workstation. The mass spectroscopy conditions are shown in table 2.11.

Table 2.11: MALDI-TOF Mass Spectroscopy Conditions

Acquisition control	Manual
Instrument mode	Linear
Extraction mode	Delayed
Polarity	Positive
Matrix	Sinapinic acid
Laser intensity	2086
Spectrum acquisition: shots/spectrum	300
Spectrum acquisition: mass range	50,000 – 100,000
Accelerating Voltage	25000 V
Voltages: grid	93%
Voltages: delay time	800 nano seconds
Guide wire 0	0.15%

2.4.1.8 Conclusion

The method development described in section 2.4.1 for the detection of CDT in serum using a HPLC-UV method from a published paper (Helander et al. 2003) proved unsuccessful. The method did not provide four identifiable peaks of each of the transferrin glycoforms under investigation. The method development resulted in another analytical method for the development of CDT being employed. The method used to analyse serum samples collected for the HI and ADI study, was the N-Latex method and is described in the following section.

2.4.2 Serum Carbohydrate-Deficient Transferrin (CDT) Analysis for HI and ADI Study Samples

This method was used as the HPLC method development was not successful in separating each of the CDT glycoforms. This sections describes the analysis of serum samples for CDT by the N Latex CDT immunoassay (Dade Behring, Marburg GmbH, Emil-von-Behring-Str. 76 35041 Marburg, Germany).

The CDT analysis was conducted within the immunology department, Northern General Hospital in Sheffield, with the kind support and collaboration of Dr. Graham Wild.

2.4.2.1 Sample Shipment

Serum samples were thawed from -80 ℃ and 600 µl of serum was aliquoted into labelled eppendorf tubes. Samples were blinded for analysis within the immunology department. Serum samples were shipped within 24 hours on dry ice by courier from QMU to the Northern General Hospital in Sheffield.

2.4.2.2 Principles of Method

Daily alcohol consumption of 50-60 g over a two week period can lead to increase in CDT levels (Helander et al. 2003). After a period of two week abstinence, CDT levels can return to normal; however this is dependent upon the initial increase of CDT levels, thus the higher the level the longer it will take to normalise.

The N Latex CDT kit is a competitive immunoassay where CDT present in the serum sample competes with CDT coated polystyrene particles, bind with specific monoclonal antibodies of human CDT, which are likewise bound to polystyrene particles. The binding of the CDT particles to the antibodies generates a light signal which can be measured and quantified. In the presence of CDT within the serum sample there is either no or little aggregation of the polystyrene particles. In the absence of CDT in the sample, the polystyrene particles aggregate. The higher the CDT content in the assay, the lower the scattered light signal. The elevation of the results is performed by comparison with a standard of known concentrations, which is provided within the kit.

The reference curves are generated by multi-point calibration with the reagents provided within the N Latex CDT kit and the resulting calibration curve is automatically generated by the software within the BN ProSpec System© (Dade Behring). The reference curves are valid for two weeks, but must be repeated if new reagents from different kit batches are used. The calculation of %CDT is automatically performed using the software integrated within the BN ProSpec System©.

2.4.3 Homocysteine in Plasma

Plasma homocysteine was analysed using High Pressure Liquid Chromatography (HPLC) with Electrochemical Detection (ED), in all samples from the HI and ADI studies.

2.4.3.1 HPLC Apparatus

The chromatography system consisted of a Waters 1525 Binary HPLC (High Pressure Liquid Chromatography) Pump (Waters Limited, 730-740 Centennial Court, Centennial Park, Elstree, Hertfordshire, WD6 3ZS, UK) and a Waters 717 plus Autosampler (Waters). Detection of the homocysteine thiol function was carried out on a Waters 2465 Electrochemical Detector (Waters) consisting of a 3mm Gold (Au) reference electrode (Waters). The electrochemical detector was connected to a Dell computer which controlled the HPLC data acquisition and integration. Separation and quantification of homocysteine was performed on a Gemini-NX 5u C_{18} column (150 x 4.6 mm) (Phenomenex UK Ltd, Melville House, Queens Avenue, Hurdsfield Industrial Estate, Macclesfield, Cheshire SK10 2BN, UK). A precolumn was not used prior to homocysteine separation on the Gemini-NX C_{18} column, however guard columns were used to protect and increase the lifespan of the Gemini-NX C_{18} column.

2.4.3.2 Preparation of Calibration Curve

For each batch of samples analysed, a calibration curve was produced. The calibration curve was prepared using dilutions from a stock solution of HPLC grade homocysteine (Sigma). Homocysteine was diluted within mobile phase. The mobile phase for this HPLC analysis consisted of 1% phosphoric acid, 10% methanol, 2 mM potassium chloride and 1 mM sodium octylsulphate (SOS). Six concentrations were adopted for the study in light of the evidence provided by Mansoor et al. 1992, which detailed the average homocysteine concentration found in human plasma. The calibration curve represented a concentration range of 0%, 50%, 100%, 150% and 200% of the expected normal levels of homocysteine found in plasma.

2.4.3.3 Preparations of Samples

This method for the detection of homocysteine in plasma was developed from a pre-existing HPLC method, which had been previously validated at Queen Margaret University (Cummins 2005).

Plasma samples were thawed from -80 ºC and processed in duplicate. 200 µl of plasma was added to 200 µl of 1 mM EDTA (Sigma) and 20 µl of Octanol-1 (Sigma), which acted as an anti-foaming agent. 100 µl of sodium borohydride (Sigma), a potent reductant was added and the solution was then vortexed and incubated at 50 ºC for 30 minutes in a water bath (Waterbath Shaker SBS30, Stuart Scientific, Beacon Road, Stone, Staffordshire, ST15 0SA, UK). $NaBH_4$ (Sigma) was prepared immediately before use by adding 74 mg $NaBH_4$ (Sigma) to 10 ml EDTA (Sigma) containing 140 µl of 10M NaOH (Sigma). 100 µl of 1M perchloric acid (BDH Laboratory Supplies, Poole, England BH15 1TD) was added to precipitate proteins (Hobbs et al. 2005). The use of perchloric acid to precipitate protein, prior to homocysteine analysis by HPLC-ED, has been established in the literature. The average amount of protein remaining in the plasma supernatant after perchloric acid precipitation is approximately 1.2 mg/ml (Jacobsen et al. 1989). Within normal human plasma, homocysteine at a mean concentration of 16 nmol/ml represents approximately 4% of the total soluble thiol reactivity after reduction by sodium borohydride and perchloric acid precipitation (Jacobsen et al. 1989). This evidence from the literature suggests that perchloric acid is approximately 96% efficient at protein precipitation (Jacobsen et al. 1989). Samples were vortexed (WhirliMixer, Oldmixon Crescent, Weston-Super-Mare, North Somerset BS24 9BL) and centrifuged (MSE Micro Centaur Microcentrifuge, Sanyo Biomedical, Sanyo Commercial Solutions, 1300 Michael Drive, Suite A, Wood Dale, IL 60191 USA) at 13,000 rpm (15,000 *g*) for 30 minutes to separate the precipitated proteins and the supernatant. The supernatant was transferred to a 1 ml clear glass vial suitable for the auto-sampler injector. A volume of 20 µl was injected into the HPLC system. Separation of homocysteine was achieved isocratically using an aqueous mobile phase.

2.4.3.4 Mobile Phase

The mobile phase was developed from the work by Cummins (2005) and the Waters 2465 Electrochemical Detector Guide. The aqueous mobile phase consisted of: 2 mM potassium chloride (Sigma), 1% phosphoric acid, (BDH) 10% methanol (Fisher Scientific) and 1 mM sodium octylsulphate (SOS) (Sigma). The pH of the mobile phase was adjusted to 1.75 using 10M NaOH (Sigma). The mobile phase was

degassed under vacuum in a bath type sonicator (Deacon DS100b, Decon Laboratories Ltd, Hove, East Sussex, U.K.).

2.4.3.5 Running Conditions

HPLC was performed at room temperature. Flow rate was 0.8 ml per minute at +0.86 volts (V) with a range of 500 nanoAmps (nA). The oxidation potential of +0.86 volts was used, as this was the oxidation used in the original method by Houze et al. (2001) and from the method which was established by Cummins (2005) at QMU. Under these conditions the homocysteine peak eluted at approximately 9.40 minutes, with a total run time of 15 minutes.

2.4.3.6 Validation of Homocysteine HPLC Method

2.4.3.6.1 Linearity

A calibration curve was generated for homocysteine, using a stock solution prepared from commercially available homocysteine (Sigma) and diluted with mobile phase as described in section 2.4.3.4. From a review of the literature, six concentrations in a range were adopted for the calibration. The concentrations were 0%, 25%, 50%, 100%, 150% and 200% of the normal level of homocysteine found in human plasma (Mansoor et al. 1992).

2.4.3.6.2 Precision

For the homocysteine assay the precision was determined by analysing two standard concentrations of homocysteine in one run. The samples were the standards (25% and 200%) which represent the low and high range of the calibration curve. The 25% and 200% standards were analysed in a single run, with three replicate injections per sample. A percentage coefficient of variation (%CV) was calculated to determine the intraassay precision.

The precision of the interassay was also established. The low (25%) and high (200%) standards were analysed in two different runs, which were undertaken on

different days. A percentage coefficient of variation (%CV) was calculated to determine the interassay precision.

2.4.3.6.3 Statistical Analysis

All data was analysed using Microsoft Office Excel (version 2003). Calibration curves were plotted by linear regression and an equation was calculated for the regression curve.

2.4.4 Homocysteine in Urine

Homocysteine in urine was analysed using High Pressure Liquid Chromatography (HPLC) with Electrochemical Detection (ED), in all samples from the HI and ADI studies.

2.4.4.1 HPLC Apparatus

The chromatography system consisted of a Waters 1525 Binary HPLC (High Pressure Liquid Chromatography) Pump (Waters) and a Waters 717 plus Autosampler (Waters). Detection of the homocysteine thiol function was carried out on a Waters 2465 Electrochemical Detector (Waters) consisting of a 3 mm Gold (Au) reference electrode (Waters). The electrochemical detector was connected to a Dell computer which controls the HPLC data acquisition and integration. Separation and quantification of homocysteine was performed on a Gemini-NX 5u C_{18} column (150 x 4.6 mm) (Phenomenex).

2.4.4.2 Preparation of Samples

The identification of homocysteine in urine was developed from the method published by Thomson and Tucker (1986).

Urine samples were collected into a 30 ml universal containers (Fisher Scientific) and stored at -20 ºC until analysis. Literature evidence suggest that urine, which will be subsequently analysed for homocysteine using HPLC-ED, can be stored without

the addition of acid to stabilise the thiols (Kusmierek et al. 2006; Thomson and Tucker 1986). Urine samples were thawed from -20 ºC. Urine (5 ml) was aliquoted into a centrifuge tube (Fisher Scientific) and 1 ml of 2M perchloric acid (BHD) was added to the centrifuge tube and mixed well, to prevent thiol oxidation. Samples were centrifuged (Jouan) at 2000 rpm (500 *g*) for 5 minutes. Supernatant (1 ml) was transferred, into a 1 ml clear glass vial suitable for the autosampler injector. A volume of 20 µl was injected into the HPLC system. Separation of homocysteine was achieved isocratically using an aqueous mobile phase.

2.4.4.3 Mobile Phase

The mobile phase was developed from the work of Cummins (2005) and the Waters 2465 Electrochemical Detector Guide. The aqueous mobile phase consisted of: 2 mM potassium chloride (Sigma), 1% phosphoric acid (BDH), 10% methanol (Fisher Scientific) and 1 mM sodium octylsulphate (SOS) (Sigma). The pH of the mobile phase was adjusted to 1.75 using 10 M NaOH (Sigma). The mobile phase was degassed under vacuum in a bath type sonicator (Decon).

2.4.4.4 Running Conditions

HPLC was performed at room temperature. Flow rate was 0.8 ml per minute at +0.86V with a range of 500 nanoAmps (nA). Under these conditions the homocysteine peak eluted at approximately 9.40 minutes, with a total run time of 15 minutes.

2.4.4.5 Creatinine in Urine

The measurement of homocysteine in urine is expressed as a ratio with creatinine, (Thomson and Tucker 1986). Creatinine is used as comparative value when expressing homocysteine as the concentrations of creatinine excreted by each individual is relatively constant and allows the concentration of homocysteine to be normalised against a constant (Thomson and Tucker 1986).

The creatinine assay was calibrated using known standards prior to analysis of the study urine samples. A calibration curve was generated for creatinine using three standards, which were provided within the Infinity™ reagent kit. The three concentrations, within the commercial kit, which were adopted for the calibration curve, were: 0.01 mg/ml, 0.03 mg/ml and 0.1 mg/l. For each batch analysed the calibration curve was linear, with a coefficient of determination of greater than 0.99. The concentration of creatinine was determined from the calibration curve.

Creatinine was determined using the Infinity™ Creatinine Liquid Stable Reagent (Thermo Electron Corporation, Thermo Fisher Scientific Inc. 81 Wyman Street, Waltham, MA 02454). Urine (30 µl) was aliquoted into each well on a 96 well-plate (Greiner Bio-One). 300 µL of Infinity™ Creatinine Liquid Stable Reagent was added to each well and the plate was analysed on a plate reader at 550 nm. Samples were analysed in duplicate.

2.4.5 MTHFR$_{(C677T)}$ Polymorphism

2.4.5.1 DNA Extraction

DNA Extraction was undertaken according to published methods (Rudbeck and Dissing 1998; von Ahsen et al. 1999). Anticoagulated blood (5 µl) was aliquoted into sterile 1.5 ml eppendorf tubes (Fisher Scientific) and 1 ml of PCR grade sterile water (Fisher Scientific) was added. Samples were centrifuged at 9600 rpm (12,000 *g*) for 1 minute with a microcentrifuge (MSE Micro Centaur Microcentrifuge, Sanyo Biomedical, Sanyo Commercial Solutions, 1300 Michael drive, Suite A, Wood Dale IL 60191 USA) and the supernatant was removed. The remaining pellet was lysed by the addition of 20 µl of 0.2 mol/l NaOH (Fisher Scientific), vortex mixed thoroughly and incubated at room temperature for 5 minutes. 180 µl of 0.04 mol/l Tris-HCl buffer (Sigma) were added to restore a pH of 7.5. Extracted DNA solution was stored at -80 ºC awaiting, spectrophotometric analysis followed by the Real-Time Polymerase Chain Reaction (PCR).

2.4.5.2 Spectrophotometric Analysis

Extracted DNA solution was analysed using a NanoVue Spectrophotometer (GE Healthcare, Pollards Wood, Nightingales Lane, Chalfont St, Giles, Bucks, HP8 4SP), at Napier University, Edinburgh. Neat DNA solution (2 µl) was placed on the reader plate and the sample was analysed. The spectrophotometer provided the absorbance of the sample at 260 nm, which allowed protein contamination to be identified. The analysis of the DNA sample using the NanoVue spectrophotometer (GE Healthcare), calculated the volume of DNA required for PCR analysis, which was important to achieve an identifiable PCR analysis for the $MTHFR_{(C677T)}$ polymorphism.

2.4.5.3 DNA Quality

The fragile nature of DNA can in certain cases cause extraction from a biological fluid to be unsuccessful. Many external sources of contamination e.g. high temperatures can cause DNA to degrade, resulting in the samples being unsuitable for PCR amplification. To ascertain the DNA samples (which were extracted in section 2.4.5.1) were not degraded and were of a suitable quality, an accuracy experiment on the extracted DNA was first performed. This accuracy experiment confirmed that the extracted DNA was suitable for Real-Time PCR. Using conventional PCR, the presence of the housekeeping gene Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) was identified in the DNA of two randomly selected participants. GAPDH was chosen as an accuracy marker as this gene is present in every cell and its presence indicates that DNA is feasible and present.

GAPDH sense and antisense primers (Sigma), were stored at -20 ºC and centrifuged (Mikro 200, Hettich, Seestrasse 204a, CH-8806, Bäch, Germany) at 2000 rpm (1000 g) for 2 minutes prior to use. GAPDH primers were diluted 1 in 10 with diethylpyrocarbonate (DEPC) water. The following master mix was prepared as shown in table 2.12.

Table 2.12: GAPDH PCR Master Mix (N= No of samples + 1)

Reagent	Volume (µl)	N (µl)
GAPDH Sense Primer	1	4
GAPDH Anti-Sense Primer	1	4
Sterile PCR-grade H_2O	18	90
Total	20	98

To a 0.5 ml PCR reaction tube containing a PureTaq Ready-To-Go PCR bead (GE Healthcare), 20 µl of master mix was added and mixed well. A 5 µl aliquot of the appropriate DNA was added to each labelled PCR tube. For the negative control 5 µl of sterile water (Baxter) replaced the DNA sample.

Each sample was amplified in the PCR thermal cycler to produce GAPDH cDNA, using the program shown in table 2.13.

Table 2.13: GAPDH PCR Program

Parameter	Denaturation	Cycling			Elongation	Cooling	
Cycles	1	25			1	1	
Segment	1	1	2	3	1	1	2
Target (ºC)	94	95	60	68	68	20	4
Hold (hh:mm:ss)	00:2:00	00:00:30	00:10:00	00:02:00	00:07:00	00:01:00	08:00:00

2.4.5.4 Gel Electrophoresis

After PCR amplification, the resulting GAPDH cDNA was examined using gel electrophoresis. The presence of GAPDH cDNA at 500 base pairs is indicative of a positive result, where GAPDH has been successfully amplified and the original DNA was of suitable quality to permit PCR amplification.

The gel electrophoresis method by Lewis 2009 was used. Agarose (0.5 g) (Invitrogen) was weighed out into a 250 ml conical flask, and 50 ml of 0.5X Tris Borate EDTA (TBE) (Sigma) buffer was added. The flask was swirled to mix and

was placed in a microwave at full power (800 watts) for 1 minute to allow the agarose to dissolve. The solution was allowed to cool for 5 minutes, within a fume cupboard, 1 µl of Ethidium bromide (Sigma) was added. Ethidium bromide is added to the solution, as it binds to DNA to allow the PCR bands to be viewed under ultra-violet light. The agarose solution was poured into an electrophoresis tank (Bio-Rad) and allowed to set for 30 minutes. Once the gel had set, 250 ml of 0.5X TBE buffer was poured into the tank to submerge the gel. The TBE buffer acted as a running buffer.

PCR products were prepared in 0.5 ml PCR reaction tubes (GE Healthcare), prior to gel electrophoresis. A 10 µl PCR product was aliquoted into each PCR reaction tube and 2 µl of loading buffer was added. A 100 base pair DNA ladder (Sigma) was also prepared, where 10 µl of the DNA ladder was aliquoted into a PCR reaction tube and 2 µl of loading buffer was added. All samples were mixed well and 12 µl of each sample and 12 µl of DNA ladder were dispensed into each well within the gel. Gel electrophoresis was carried out at 100 volts for 30 minutes. To view cDNA bands, the gel was photographed under UV light, using a Syngene Imager (Syngene, Beacon House, Nuffield Road, Cambridge, United Kingdom, CB4 1TF).

2.4.5.5 Preparation of Samples

$MTHFR_{(C677T)}$ primers were purchased from Tib MolBiol (GmbH. Berlin Germany). Primers were stored at room temperature and protected from light. The PCR reaction is highly sensitive, results can be obtained from 1 ng of genomic DNA and generated within 1 hour (Rudbeck and Dissing 1998).

Reagent vials containing all primers and probes were removed from the Tib MiolBiol kit and 66 µl of PCR grade water was added to each volume. The solutions were vortexed and centrifuged (Hettich). A 4 µl volume of primer and probe reagent were sufficient for a 20 µl PCR reaction, for each sample being analysed.

Due to the small volumes used in a PCR reaction, a master mix was used to reduce pipetting errors. To create a master mix, the kit, LightCycler FastStart DNA Master

HybProbe was purchased from Roche (Roche Applied Science, 68298 Mannheim, Germany). A reaction tube was cooled to 4 ºC and the master mix was prepared by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction. The reagents used in the master mix are detailed in table 2.14.

N (volume required) = number of participant samples + 1 x reagent volumes

Table 2.14: $MTHFR_{(C677T)}$ PCR Reaction Master Mix

Master Mix Reagent	Volume (µl)
PCR grade Water	7.4
Mg^{2+} Solution 25 mM	1.6
Reagent Mix	4.0
FastStart Mix	2.0
Total Volume	**15**

Reaction master mix was mixed gently, centrifuged (Jounan) and 15 µl was transferred to a LightCyler capillary (Roche). Participant DNA samples or DNA control (5 µl) were added to each LightCycler capillary (Roche) to give a final reaction volume of 20 µl.

2.4.5.6 Control DNA

For each PCR analysis a positive and negative control was included with the participant's samples. For the positive DNA control a template DNA sample was provided with the MTHFR kit (Tib MolBiol). PCR grade water (40 µl) was added to the control DNA vial. The sample was mixed well by pipetting the solution up and down 10 times. A 5 µl volume of the control DNA sample was required for a 20 µl PCR reaction.

A negative DNA control was prepared in the same way as the positive control, where the template DNA was replaced with PCR grade water.

2.4.5.7 Real-Time PCR Program

The $MTHFR_{(C677T)}$ Real-Time PCR program consisted of 4 steps:

1. Denaturation of sample and activation of enzyme.
2. PCR amplification of the target DNA.
3. Melting curve for identification of the genotype.
4. Cooling of the instrument.

The following program shown in table 2.15 was programmed into the LightCycler software (Roche) to detect the $MTHFR_{(C677T)}$ polymorphism:

Table 2.15: $MTHFR_{(C677T)}$ Real-Time PCR Program

Parameter	Denaturation	Cycling			Melting			Cooling
Analysis Mode	None	Quantification			Melting Curve			None
No of Cycles	1	45			1			1
Segment	1	1	2	3	1	2	3	1
Target (ºC)	95	95	55	72	95	40	85	40
Hold	10 mins	.05 sec	10 sec	15 sec	20 sec	20 sec	0 sec	30 sec
Ramp Rate (ºC/s)	20	20	20	20	20	20	0.2	20
Acquisition Mode	None	None	Single	None	None	None	Continuous	None

2.4.5.8 Data Analysis

The polynominal calculation method was employed for analysing the melting peaks. The Roche LightCycler software program for Real-Time PCR amplification carried out the polynominal calculation. The melting temperature was determined with the manual Tm setting. The $MTHFR_{(C677T)}$ data was viewed in channel 640 using the melting curves analysis mode. The negative control showed no signal, due to no presence of DNA within the sample.

2.4.6 Serum Combined Folate and Vitamin B_{12} Assay

Within participant's serum samples, folate and vitamin B_{12} analyses were conducted within the biochemistry laboratories at the Royal Infirmary of Edinburgh (RIE). The assay was conducted by the laboratory biomedical staff. Folate and vitamin B_{12} are analysed as a joint assay in duplicate.

Serum samples were thawed from -80 ºC to room temperature (20 ºC) and 800 µl of serum was aliquoted into chilled 5 ml centrifuge tubes (Sarstedt). Centrifuge tubes were labelled with barcodes, provided by the biochemistry laboratories at RIE. Samples were transferred to RIE on wet ice.

2.4.6.1 Folate Analysis

Folate was analysed within serum using the ADVIA Centaur Folate assay, which is a competitive immunoassay, using direct chemiluminescent technology (Bayer HealthCare LLC 2006). The principle of this method involves the sample being pretreated to release the folate from endogenous binding proteins in the sample. The results from this assay are based on the inverse relationship between the amount of folate present in the participant sample and the amount of relative light units (RLU), detected by the system. The sensitivity range of this assay is 0.35 - 25 ng/ml (0.79–54.36 nmol/l) (Bayer HealthCare LLC 2006) and the clinical reference range for folate in human serum is 5-24 µg/l.

2.4.6.2 Vitamin B_{12} Analysis

Vitamin B_{12} was analysed within serum using the ADVIA Centaur VB12 assay, which is a competitive immunoassay using direct chemiluminescent technology (Bayer HealthCare LLC 2004). The principle of the assay uses sodium hydroxide as a releasing agent and DTT to release the vitamin B_{12} from the endogenous binding proteins in the sample and cobinamide to prevent rebinding after the solid phase is added to the sample. The results from this assay are interpretated in a similar way to that of the folate assay where there is an inverse relationship between the amount of vitamin B_{12} in the sample and the relative light units (RLU) detected by the system. The sensitivity range of this assay is 45–2000 pg/ml (33–1476 pmol/L) (Bayer HealthCare LLC 2004) and the clinical reference range for vitamin B_{12} is human serum is 200-900 ng/l.

2.5 Summary

Participant's biological samples, questionnaire's and alcohol diaries were analysed as detailed in table 2.16 as per each method described within this chapter.

Table 2.16: Summary HI and ADI Study Analysis

Study	Plasma Homocysteine	Serum Folate	Serum Vitamin B_{12}	Serum CDT	$MTHFR_{(C677T)}$ Polymorphism	Diet & Alcohol Diary (grams of alcohol per drinking day)	Questionnaire (grams of alcohol per drinking day)
HI	•	•	•	•	•	•	•
ADI	•	•	•	•	•		•

2.6 Quality Control: Investigation of the Stability of Study Biomarkers during Storage (Stability Study)

The stability of the metabolite being analysed is a highly important consideration, impacting on the choice of methods used to collect, handle and store biological samples. Several studies have investigated the differing conditions in which samples can be stored before analysis was carried out. This study was developed to identify the effects of repeated thawing and re-freezing on the stability of homocysteine, carbohydrate-deficient transferrin (CDT), folate and vitamin B_{12}.

Plasma and serum prepared from the blood samples provided by both the HI and ADI study participants were stored, frozen until batch analysis could be performed. To confirm the stability of the various biomarkers under investigation during this time it was necessary to perform supporting stability studies.

2.6.1 Stability Study Protocol

This study required a large volume of human plasma (40 ml) and serum (80 ml). It was obtained from participants who were recruited from QMU, through e-mail advertisement. Recruitment was conducted over a week long period in May 2008.

2.6.1.1 Sample Storage

Four groups were designed for this study, each group of samples were exposed to different sets of thawing and re-freezing time-points, during a 3-month period, which are detailed in table 2.17.

Table 2.17: Storage conditions for stability study sample groups

Group Number	Conditions	Contents
1	'Spiked' Samples at Constant -80 ºC	• 10 x 1 ml Homocysteine spiked plasma • 10 x 1 ml CDT spiked serum • 10 x 1 ml Folate/Vitamin B_{12} spiked serum
2	'Spiked' Samples Freeze/Thaw from -80 ºC on 3 occasions	• 10 x 1 ml Homocysteine spiked plasma • 10 x 1 ml CDT spiked serum • 10 x 1 ml Folate/Vitamin B_{12} spiked serum
3	Samples at Constant -80 ºC	• 10 x 1 ml Homocysteine plasma • 10 x 1 ml CDT serum • 10 x 1 ml Folate/Vitamin B_{12} serum
4	Samples Freeze/Thaw from -80 ºC on 3 occasions	• 10 x 1 ml Homocysteine plasma • 10 x 1 ml CDT serum • 10 x 1 ml Folate/Vitamin B_{12} serum

For each metabolite there were ten 1 ml samples (eppendorfs) stored in each group. Within groups 1 and 2 plasma and serum samples were 'spiked' with artificial standards (described in section 2.6.1.4), which were commercially available. Folate in the form of folic acid and Vitamin B_{12} were purchased from Sigma. CDT Test Mix were purchased from Bio-Rad.

2.6.1.2 Thawing and Re-Freezing Procedures

Group 1 and 3 samples were kept at a constant -80 ºC temperature during the 3 month duration of the study. On a monthly basis the samples within groups 2 and 4 were thawed at specific time points, as detailed in table 2.18. Each thawing time-point was one month apart. After each thawing time-point the samples were returned to the -80 ºC freezer. For the final thawing time-point, the samples were completely thawed, before being returned to -80 ℃ storage.

Table 2.18: Monthly Freezing and Thawing Time-points

Thawing Time-point	Temperature Change (ºC)	Time Period (hours)
Month 1	-80 ºC to -20 ºC	24 hours
Month 2	-80 ºC to -20 ºC	24 hours
Month 3	-80 ºC to 0 ºC	6 hours

2.6.1.3 Stability Study Venepuncture and Sample Processing

All stability study participants were asked to attend a clinic at QMU, so all venepuncture, could be undertaken at the same time. They were asked to fast for 12 hours prior to their blood sampling appointment. A fasting blood sample was taken from each participant.

Venepuncture and blood sample processing was conducted as described in section 2.1.3.6.1. All serum samples were pooled in a glass beaker (Fisher Scientific), mixed well and a 1 ml sample was aliquoted into ten 1.5 ml eppendorf tubes (Fisher Scientific) and stored at -80 ºC, awaiting CDT analysis by immunoassay and combined Folate/Vitamin B_{12} competitive immunoassay at the Royal Infirmary of Edinburgh. All plasma samples were pooled together in a glass beaker mixed well and ten 1 ml samples were aliquoted into 1.5 ml eppendorf tubes (Fisher Scientific) and stored at -80 ºC, awaiting homocysteine analysis by HPLC (Cummins 2005; Houze et al. 2001). Serum and plasma samples for the Stability Study were stored at QMU

2.6.1.4 Stability Study Standards

This section describes the preparation of the standards used to spike the pooled serum samples; which were subjected to the varying temperature manipulations as per described in table 2.18.

2.6.1.4.1 Homocysteine Standards

Homocysteine was purchased from Sigma (Sigma-Aldrich Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT). For standards a 1 litre stock solution was prepared to a concentration of 12.7 µmol/l. 0.1 g of homocysteine was dissolved in 1 litre of distilled water. The solution was mixed well and homogeneity was ensured. 10 µl of stock solution was aliquoted into each 1 ml pooled plasma sample. Each 10 µl aliquot has a concentration of 7.4 nmol/10 µl. Samples were stored according to group allocation, see table 2.17.

2.6.1.4.2 CDT Standards

A CDT Test Mix was purchased from Bio-Rad Laboratories. The CDT Test Mix was left to stand for 30 minutes at room temperature (21 ºC) before reconstitution, due to the product being supplied in lyophilized form. Distilled water (1 ml) was added to the test mix. The stopper was replaced and the reconstituted test mix was allowed to stand for 10 minutes. After 10 minutes the test mix was agitated and homogeneity was ensured. The test mix (50 µl) was added to each 1 ml serum sample and stored according to group allocation.

The test mix is commercially available from Bio-Rad and is used for calibration with the Bio-RAD %CDT HPLC kit. The test mix contains disialotransferrin, trialaotransferrin, tetrasialotransferrin and pentasialotransferrin. The text mix was used for the stability study, as it was the only way of spiking the serum samples with additional CDT glycoforms.

2.6.1.4.3 Folate Standards

Folic acid was purchased from Sigma. Folic acid was stored at 2-8 ºC and protected from sunlight. For standards a 1 litre stock solution was prepared, to a concentration of 0.75 mg/l (0.75 mg of folic acid was dissolved in 1 litre of distilled water). The solution was mixed well and homogeneity was ensured. The rationale for using 0.75 mg/l of folic acid, to spike the serum sample, was to keep the final concentration below the maximum clinical reference value of 20 µg/l and prevent the

concentration from reaching a level above the maximum cut-off value. Stock solution (10 µl) was aliquoted into each 1 ml serum sample, resulting in an added concentration of 7.5 ng/10 µl. Samples were stored according to group allocation.

2.6.1.4.4 Vitamin B_{12} Standards

Vitamin B_{12} was purchased from Sigma. Vitamin B_{12} was stored at 2-8 ºC. For standards a 1 litre stock solution was prepared, to a concentration of 35 mg/l (35 mg of Vitamin B_{12} was dissolved in 1 litre of distilled water). The solution was mixed well and homogeneity was ensured. The rationale for using 35 mg/l of vitamin B_{12}, to spike the serum sample, was to keep the final concentration below the maximum clinical reference value of 900 ng/l and prevent the concentration from reaching a level above the maximum cut-off value. Stock solution (10 µl) was aliquoted into each 1 ml serum sample, producing a final concentration of 350 ng/10 µl. Samples were stored according to group allocation

2.7 Statistical Analysis

Non-parametric statistical analysis was employed due to the small sample number, also as biological markers were not normally distributed.

2.7.1 Statistical Software

The statistical software used to conduct all statistical tests, data analysis, tables and graphs was SPSS (Version 17) and Microsoft Excel (Version 2003).

2.7.2 Test for Normality

A Shapiro-Wilk test was carried out on all data from each study conducted to test for normality.

2.7.3 Statistical Tests

All statistical tests which were conducted using SPSS, were two-tailed and with a significant p value of less than or equal to 0.05. The following non-parametric statistical tests were employed: Mann-Whitney test (for between group analysis), Friedman's test (multiple measures of three time-points), Spearman's test (for correlation analysis) and Wilcoxon test (for within pair-wise group analysis).

CHAPTER 3: RESULTS

3.1 Demographic Study Information

Participants in the Healthy Individual (HI) study (N=35) were recruited from Queen Margaret University (QMU) and Napier University in Edinburgh. Study participants for the Alcohol Dependent Individual (ADI) study (N=18) were recruited from the Ritson Clinic and Alcohol Problems Service which are an in-patient alcohol detoxification ward and an out-patient clinic within the Royal Edinburgh Hospital. The demographic information relating to each group of participants is shown in table 3.1.

Table 3.1: Comparison of demographic information from the HI and ADI studies (* denotes significance)

	HI Participants (N=35)	**ADI Participants (N=18)**	**p-value**
Age in years: median, mean (SD; range)	29, 30 (8; 20-49)	39, 41 (8.9; 26-55)	0.023*
Age Groups Percentage (N)	18-25 years 31.4% (11) 26-50 years 68.6% (24)	18-25 years 0% 26-50 years 100% (18)	<0.001*
Gender Percentage (N)	Male 34.3% (12) Female 65.7% (23)	Male 83.3% (15) Female 16.7% (3)	0.891
Female Hormonal Contraceptive Users Percentage (N)	39.1% (9)	0%	NA
Smokers Percentage (N)	11.4% (4) Female (3) Male (1)	55.56% (10) Female (2) Male (8)	0.001*
Occupation Percentage (N)	Students 74.3% (26) Employed 25.7% (9)	Not recorded by questionnaire	NA

The median age of study participants was significantly lower ($p<0.05$) in the HI study compared with the patients recruited into the ADI study. There were no participants recruited into the 18-25 year group within the ADI study, due to lack of patients of this age group being in the alcohol treatment ward during the recruitment period. There was a higher percentage of female participants, in the HI study, compared to the ADI study. None of the ADI female participants used hormonal contraception, compared with 39.1% of the females in the HI study. More ADI participants were smokers in comparison to the HI study participants ($p<0.05$). Figures 3.1 and 3.2 illustrate the CONSORT diagrams for both the HI and ADI studies.

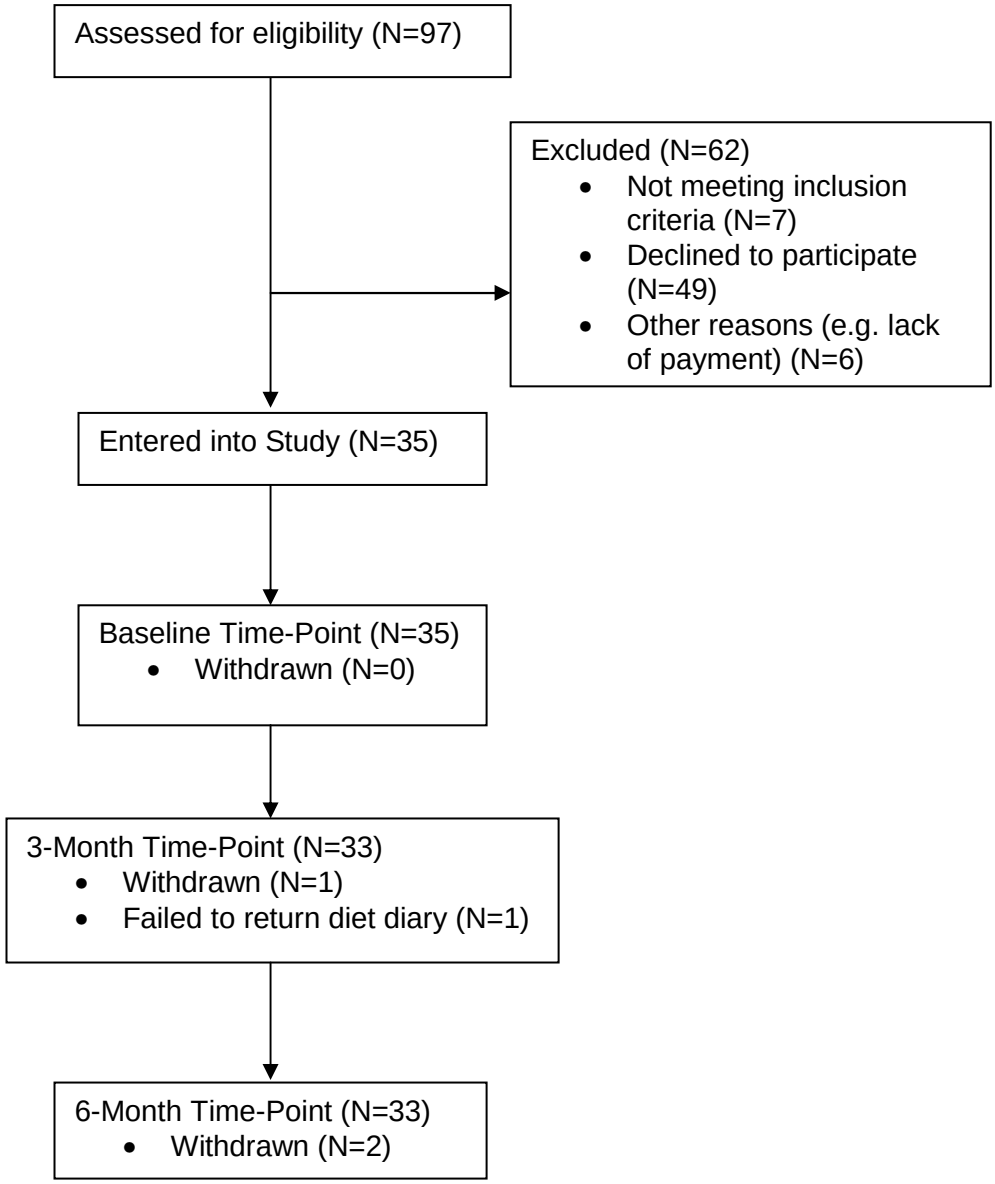

Figure 3.1: HI study CONSORT diagram

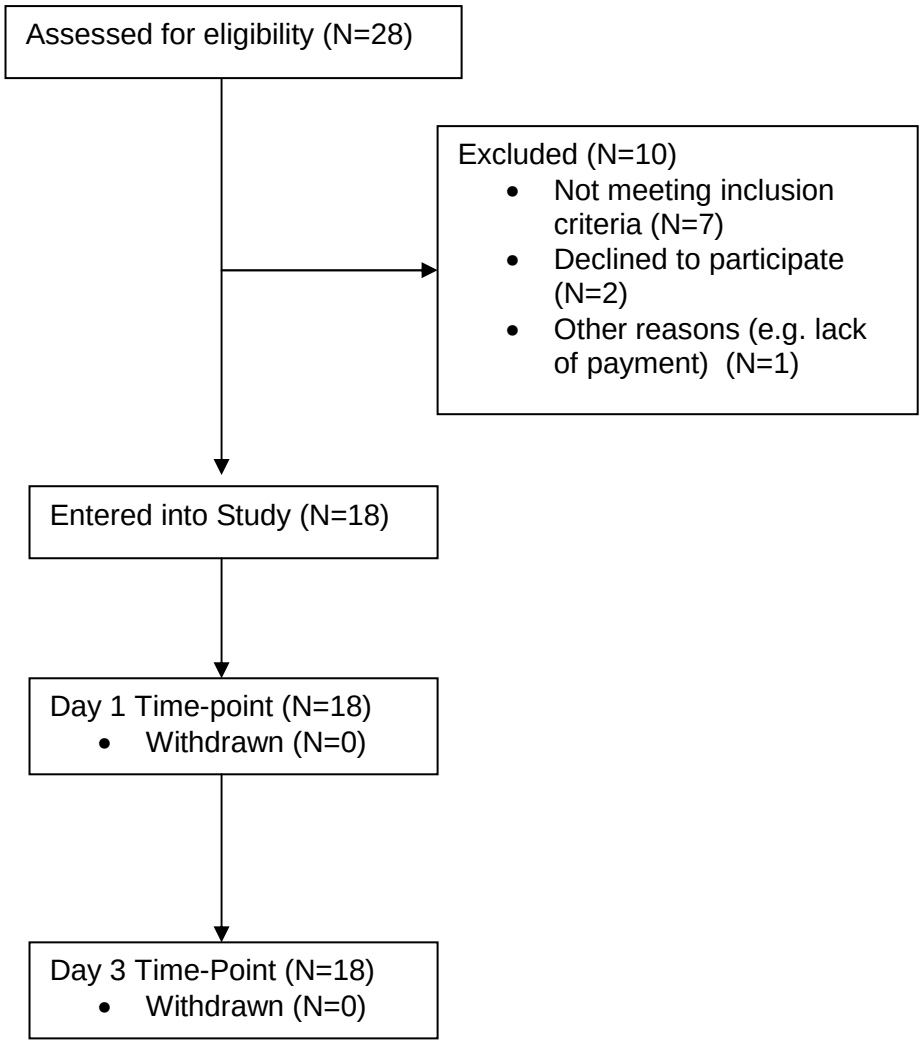

Figure 3.2: ADI study CONSORT diagram

Additionally all ADI patients (N=18) were prescribed the benzodiazepine drug, Chlordiazepoxide to reduce alcohol withdrawal symptoms. Chlordiazepoxide was prescribed using a reducing regime from admission. Both out-patients and in-patients were prescribed Chlordiazepoxide. ADI study participants were also prescribed Thiamine (N=10) or Pabrinex (N=11), with five ADI participants being prescribed both. Pabrinex and Thiamine are B-vitamin supplements, which are routinely prescribed for malnutrition. Thiamine is prescribed most commonly when malnutrition is present, however in more severe cases of malnutrition, Pabrinex is prescribed. In the ADI study, 61.6% of the sample were prescribed Pabrinex which indicates that severe malnutrition was present. Pabrinex and Thiamine supplements do not contain folic acid or vitamin B_{12}, meaning the prescribing of

these supplements did not artificially increase folate or vitamin B_{12} levels and therefore impact on subsequent test results.

3.2 Alcohol Consumption Analysis Methods used to Assess Alcohol Intake from Diary Recall (HI Study)

Baseline alcohol consumption data for 26 drinkers (18 female and 8 male), who undertook the HI study (74% of total HI sample) were analysed using three separate methods as detailed in chapter 2. Additionally two people withdrew and 7 were abstainers. The three methods employed were as follows: dietary analysis using alcohol manufacturer data (AM); WinDiets dietary analysis software (WD) and alcohol consumption quantification methods employed in the Office of National Statistics (ONS) questionnaires (Goddard 2001).

Participant's alcohol consumption during drinking days and the variability between the three methods are shown in Appendix 3A and 3B. The results of participants mean alcohol consumption during drinking days using the three methods stated above are shown in table 3.2.

Table 3.2: Mean (SD) Alcohol Consumption of all drinkers (N=26) during drinking days at baseline analysed using the ONS, WD and AM methods (*denotes significance)

	Mean (SD) Alcohol Consumption During Drinking Days (g)			
	ONS	**WD**	**AM**	**p-value**
All Participants (N=26)	33.9 (22.8)	48.6 (29.6)	53.3 (37.5)	<0.001*
Female Participants (N=18)	27.7 (21.0)	41.4 (28.1)	44.8 (34.0)	<0.001*
Male Participants (N=8)	47.7 (21.7)	65.0 (27.7)	72.4 (40.3)	0.010*

Baseline mean alcohol consumption during drinking days was calculated in grams/day. Due to the greater number of females, compared to males (18 versus 8), it was possible to further group female participant's data (N=18) according to the following drinking categories: "responsible" (<24g/day); "exceeding daily limits" (24-

47g) and “sessional” (>48g) at one session using the AM method. Their alcohol consumption at baseline was then calculated using the ONS, WD and AM methods and compared (figure 3.3). The number of female participants categorised to each drinking pattern using each of the ONS, WD or AM methods is shown in figure 3.4.

If it is assumed that consideration of specific alcohol contents and drink sizes will produce the most accurate estimate of alcohol intake (i.e. the AM method), then the WD method underestimates average consumption by 8.8% and the ONS method by 36.4%. A significant decrease ($p<0.001$) was evident for the mean alcohol consumption during drinking days analysed by the ONS method compared to AM but there was no significant difference ($p=0.258$) for the WD alcohol analysis when compared to AM.

WD and ONS miscategorised 11% and 55% of participants, respectively and is shown in Figure 3.4. A significant difference ($p=0.0001$) between the number of participants categorised to each drinking pattern using the AM and WD method was evident It is clear that female participants who were undertaking sessional drinking, calculated using the AM method, would in fact not be found to be sessionally drinking, if their diaries were analysed using the ONS method (figure 3.3)

3.2.1 Summary

Self-reported alcohol consumption data can be collected and then analysed using a number of different methods. From the results reported in this section it is clear, that the AM method is the most accurate as it takes into consideration relevant information needed to calculate alcohol consumption, including brand of alcoholic beverage, %ABV and volume. All diaries and questionnaire data relating to alcohol consumption were subsequently analysed using the AM method.

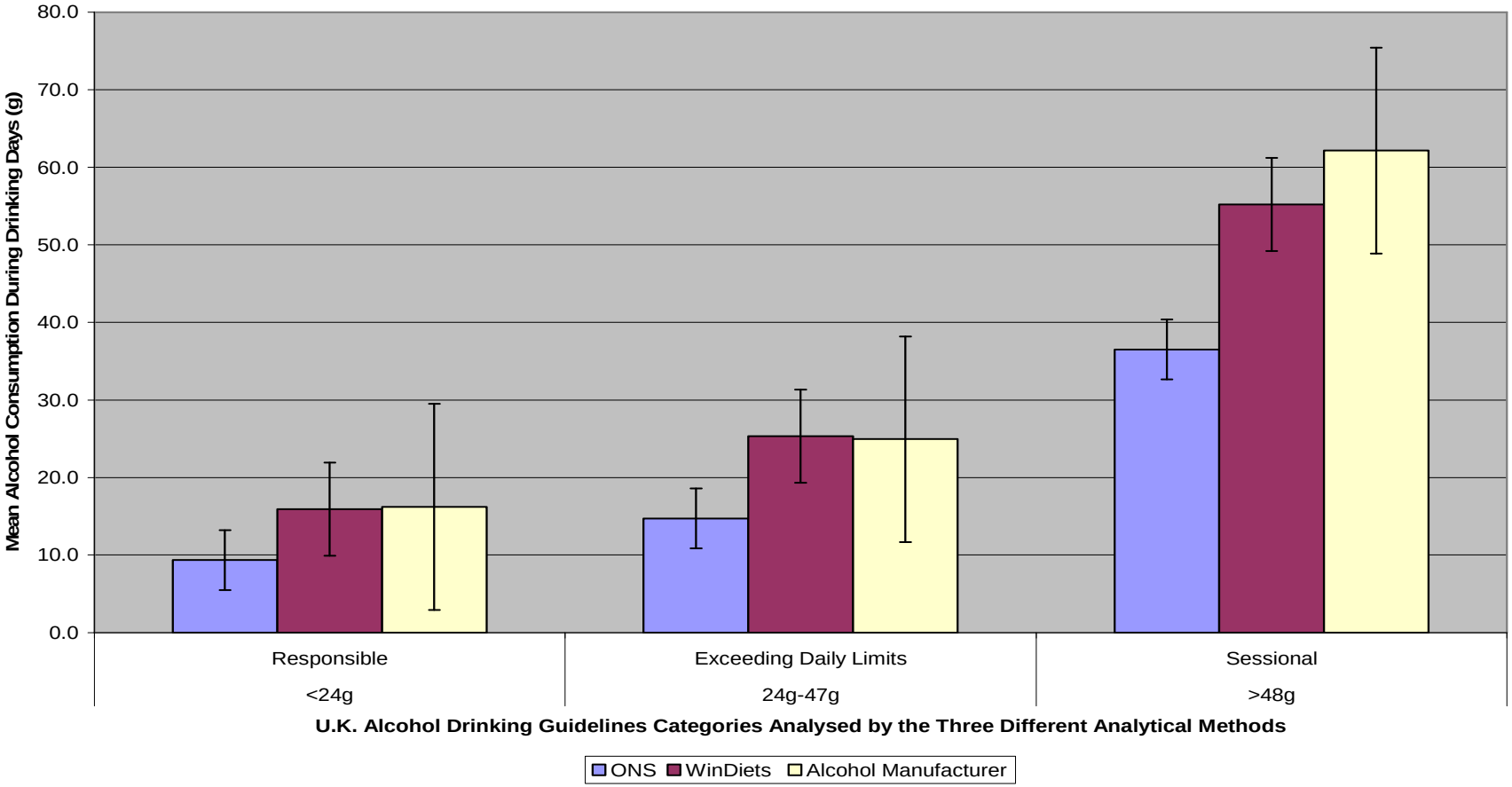

Figure 3.3: Baseline alcohol consumption analysed using AM and compared to ONS and WD of female participants categorised according to U.K. alcohol drinking guidelines (mean and standard deviation)

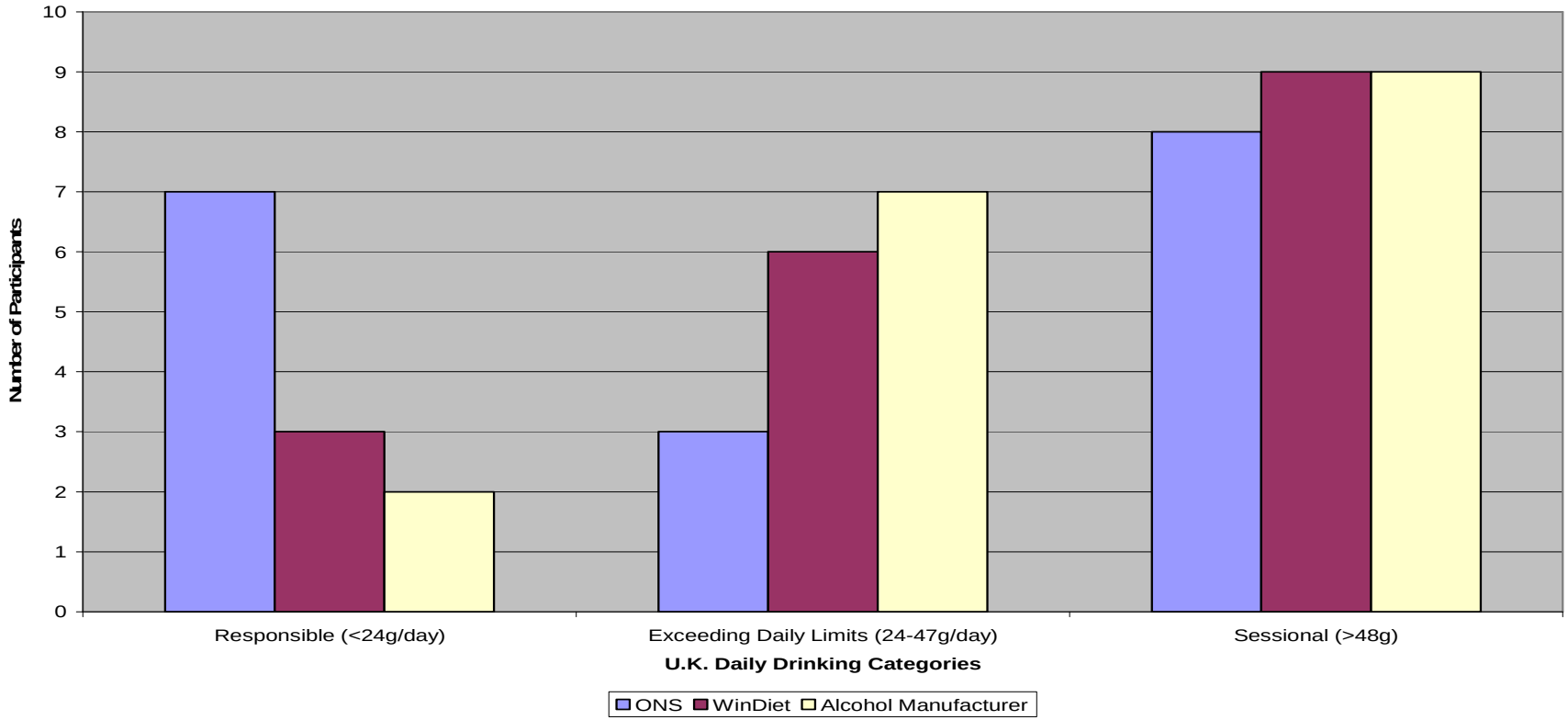

Figure 3.4: Categorisation of female participants' self-reported drinking patterns at baseline using three different calculation methods (ONS, WD and AM data)

3.3 Alcohol Drinking Patterns and Consumption

3.3.1 HI Study

A comparison of HI study participants' median alcohol consumption across time-points, suggested that alcohol consumption decreased during the 6-month study period, see figure 3.5, however this was not statistically significant (p=0.910). It is possible that the monitoring effect of the study could have influenced alcohol drinking, producing a change in normal consumption; however the lack of statistical significance is not consistent with this possibility. However all baseline biomarker data were compared to alcohol consumption levels and drinking patterns at baseline for the HI study analysis.

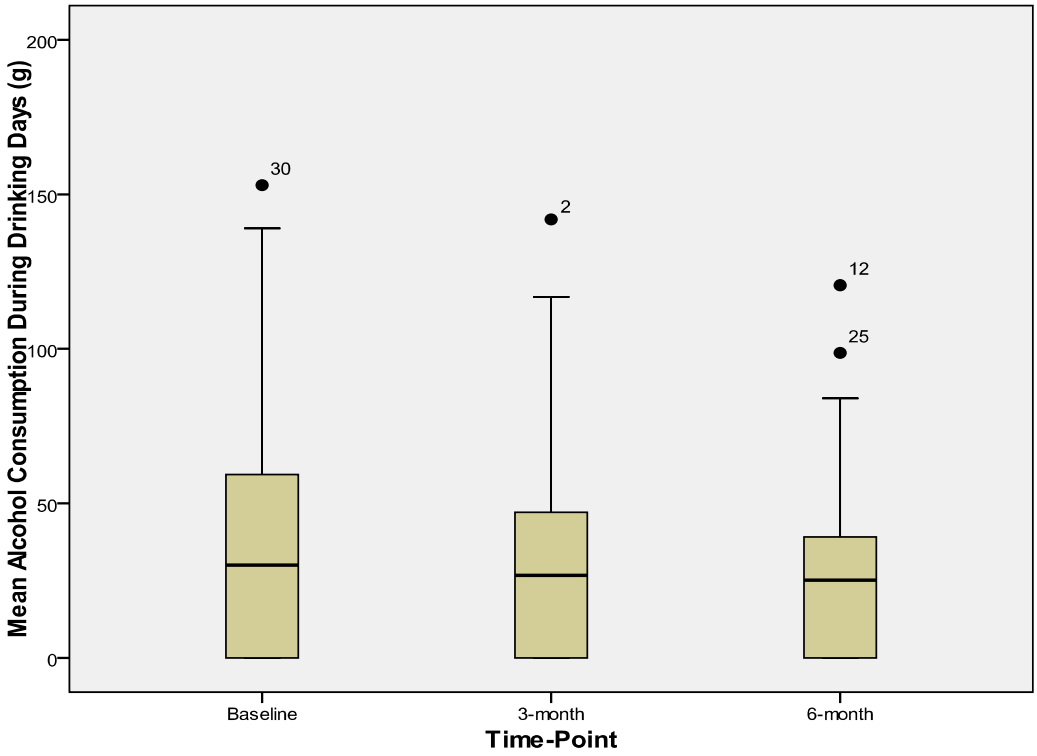

Figure 3.5: Mean alcohol consumption during drinking days of HI study participants at the pre-defined study time-points (N=35). *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

The consumption of alcohol can be categorised into alcohol drinking patterns using the recognised criteria as described in table 3.3.

Table 3.3: Alcohol drinking pattern criteria

Alcohol Drinking Pattern	Criteria
Abstainer	Consume no alcohol or less than 1-2 alcoholic drinks per year.
Responsible drinker	Consume alcohol according to UK Department of Health guidelines, not exceeding 16-24 g (female) and 24-32 g (male) per day (UK Department of Health 1995).
Exceeding Responsible Guidelines but NOT Sessional Drinkers	Consume greater than 24 g (female) and 32 g (male) of alcohol but less than sessional drinker on any drinking day.
Sessional drinker	Consume greater than 48 g (female) and 64 g (male) of alcohol on any drinking day in a typical week (UK Department of Health 2009).

HI study participants were asked if they would consume more than 6 (48 g; female) or 8 (64 g; male) units of alcohol in a session, which is the definition for sessional drinking, 53.6% (N=14) of the HI study sample answered “yes” to this question. As shown in table 3.4, from analysis of the baseline diet diaries, 18 (51.4%) HI study participants were found to undertake sessional drinking, which indicates that four HI study participants did not self-report their sessional drinking. The demographic characteristics of the HI study participants, who were categorised according to their baseline diaries, are shown in table 3.4. Figure 3.6 representing the grams of mean alcohol consumed per drinking day within each alcohol drinking pattern at the three pre-defined study time-points, determined from baseline diaries. The abstaining individuals (N=7) were identified from the study questionnaire and confirmed by diary analysis. The median value for the mean alcohol consumption per drinking days in the sessional drinkers group did not significant decrease (p=0.642) at each pre-defined study time-point.

Table 3.4: Demographic study information of all HI study participants (N=35) categorised according to alcohol drinking pattern using baseline diet diaries. *denotes significant difference between groups.

	Abstainer (N=7)	**Responsible Drinkers (N=2)**	**Exceeding Responsible Guidelines but NOT Sessional Drinker (N=8)**	**Sessional Drinkers (N=18)**	**p-value**
Age (years): (Median; Mean (SD; Range))	32; 33 (10; 20-45)	23; 23 (1; 22-23)	30; 33 (8; 26-49)	28; 30 (8; 21-46)	0.120
Age Groups Distribution, Percentage (N)	18-25 yrs 28.6% (2) 26-50 yrs 71.4% (5)	18-25 yrs 100% (2) 26-50 yrs 0%	18-25 yrs 0% 26-50 yrs 100% (8)	18-25 yrs 38.9% (7) 26-50 yrs 61.1% (11)	0.002*
Gender Distribution Percentage (N)	Male 57.1% (4) Female 42.9% (3)	Male 0% Female 100% (2)	Male 10% (1) Female 90% (7)	Males 38.9% (7) Females 61.1% (11)	0.002*
Regular Period in Females Percentage (N)	33.3% (1)	50% (1)	85.7% (6)	81.8% (9)	0.012*
Female (N=23) Hormonal Contraceptive Users Percentage (N)	33.3% (1)	50% (1)	16.6% (1)	54.5% (6)	0.040*
Distribution of Smokers Percentage (N)	0%	0%	12.5% (1)	16.6% (3)	0.317
Distribution of Occupation Percentage (N)	71.4% Students (5) 28.6% Employed (2)	100% Students (2) 0% Employed	50% Students (4) 50% Employed (4)	83.3% Students (15) 16.6% Employed (3)	0.002*
Percentage of Individuals who stated they would consume >6 (female) or >8 (male) units in a session (Self-reported from questionnaire)	0%	50% (1)	12.5% (1)	77.8% (14)	<0.001*

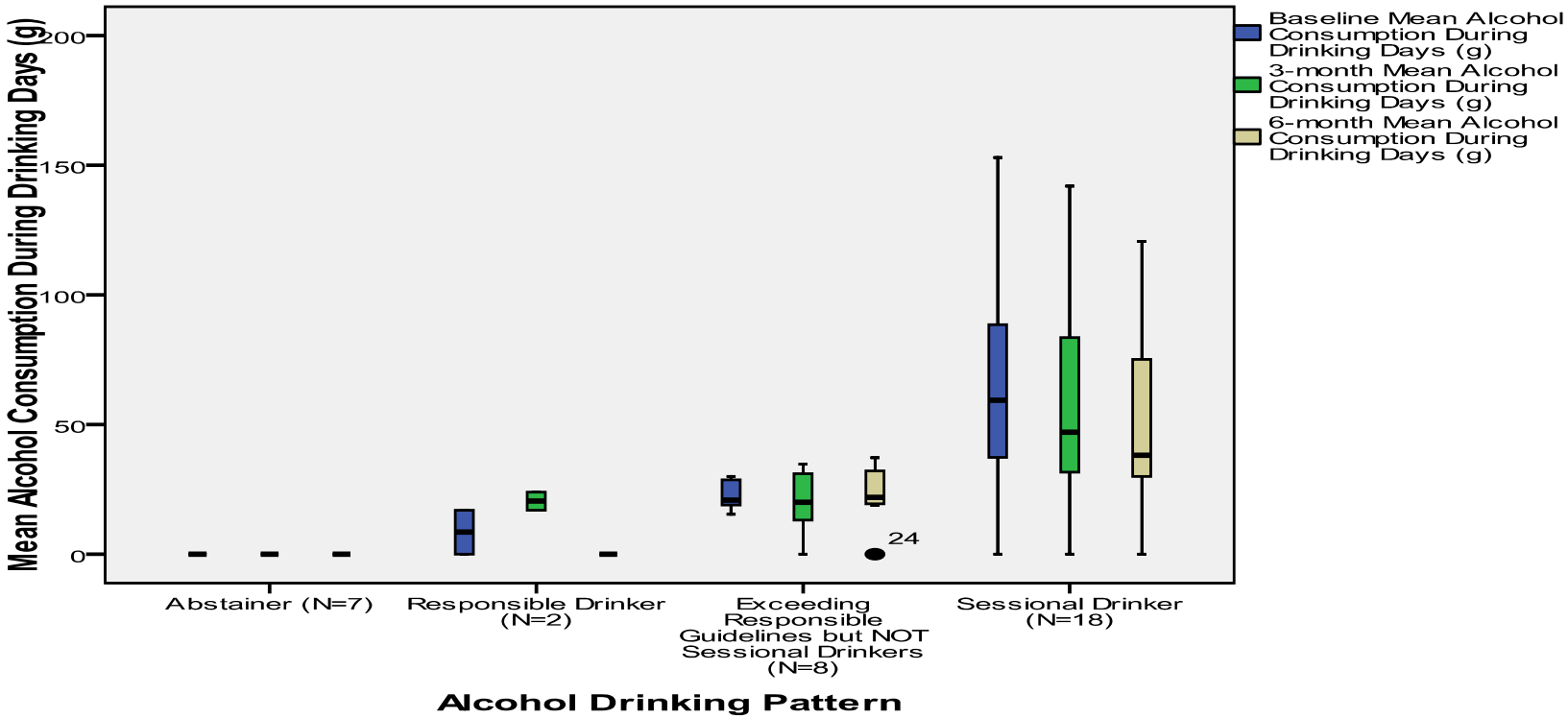

Figure 3.6: Box plots of mean alcohol consumption (g) during drinking days in HI study sample (N=35) during the course of the study. For each of the alcohol drinking patterns, data are presented for the three time-points; baseline, 3-months and 6-months. *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box).*

The number of drinking days at each pre-defined study time-point was recorded for each HI study participant and categorised using the prospective diary, reported in table 3.5.

Table 3.5: HI study participant's number of drinking days at each pre-defined study time-points, categorised according to alcohol drinking pattern (N=35)

No of Drinking Days per Week at each study time-point	**Abstainer (N=7)**	**Responsible Drinker (N=2)**	**Exceeding Responsible Guidelines but NOT Sessional Drinker (N=8)**	**Sessional Drinker (N=18)**
Baseline (Median; Mean (SD; Range))	__	0; 0 (1; 0-1)	3; 4 (2; 2-6)	5; 4 (2; 0-7)
3-Month (Median; Mean (SD; Range))	__	1; 1 (0; 1-1)	2; 2.3 (1.6; 0-5)	3; 2.8 (1.9; 0-7)
6-Month (Median; Mean (SD; Range))	__	__	4; 4.3 (2.3; 0-7)	2.0; 2.7 (2.1; 0-7)

Figure 3.7 presents the number of HI study participants and their specific alcohol drinking pattern at each time-point. At the 3-month time-point, 13 participants had changed their alcohol drinking patterns. At the 6-month time-point, 12 participants had changed their alcohol drinking patterns. The number of sessional drinkers decreased over the study period. Additionally five individuals changed their alcohol consumption pattern to consuming no alcohol in the week prior to blood sampling at the three and six month time-points. Figure 3.7 demonstrates the potential influence of the study on the drinking patterns of study participants.

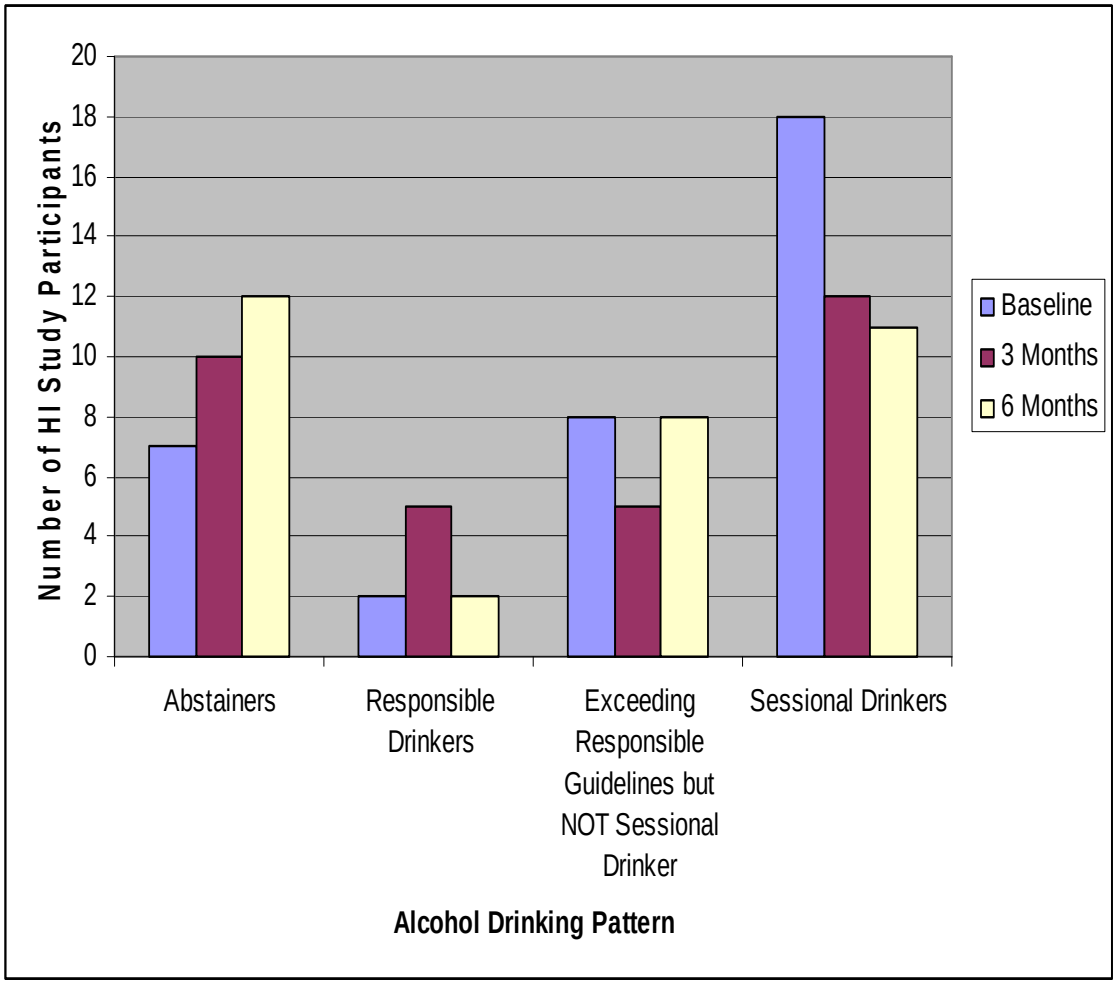

Figure 3.7: HI Study participants' (N=35) alcohol drinking patterns during study period.

3.3.2 HI Study Participants who Consume Alcohol in a Sessional Pattern

The HI study participants were sub-grouped to investigate only sessional drinkers (N=17). The consumption of alcohol by sessional drinkers was investigated and the number of days for which sessional alcohol consumption was undertaken at baseline and compared to the number of non-sessional drinking days (consumption of less than 48 g (females) and 64 g (male)) and the number of alcohol-free days, as shown in figure 3.8.

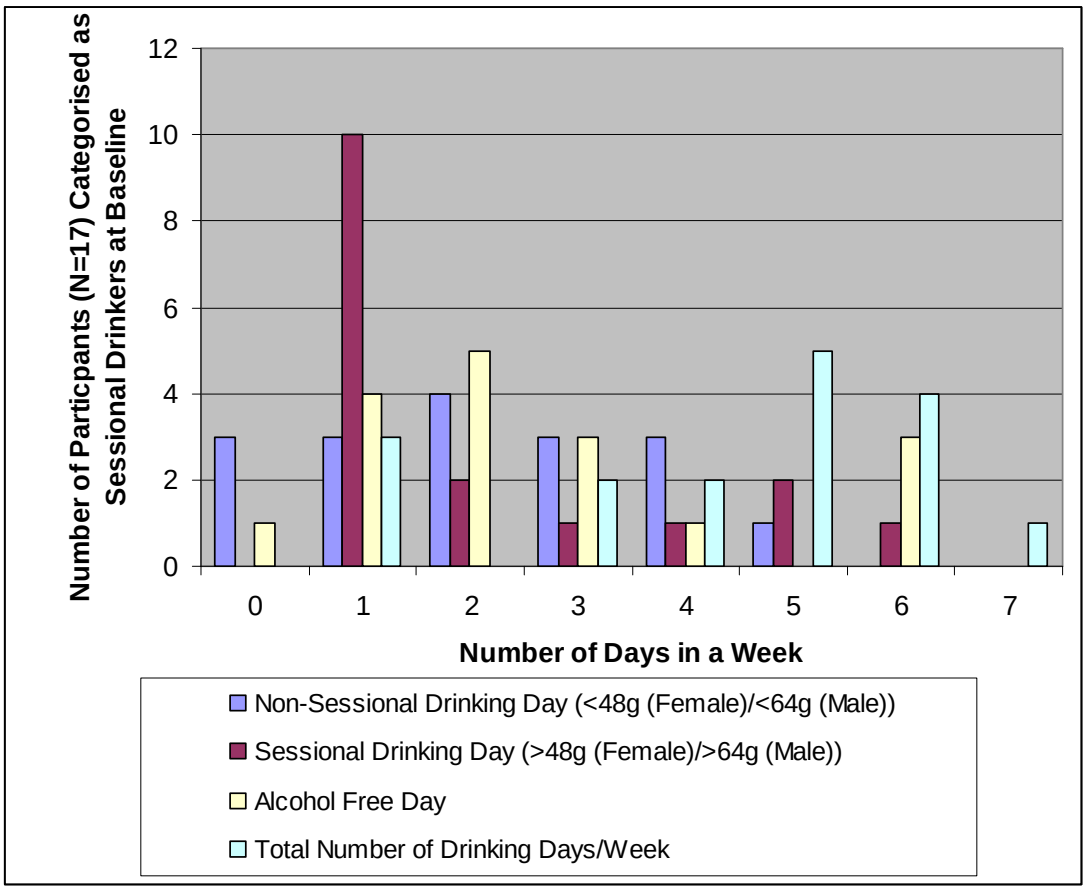

Figure 3.8: Alcohol consumption compared in a sample of sessional drinkers (N=17)

From figure 3.8, it is clear that sessional alcohol consumption was most commonly undertaken on one day within a week, within this sample, and is further illustrated in figure 3.9, where the grams of alcohol consumption on day 2 has the highest median value and range. Day 2 had the largest range of grams of alcohol consumed and the highest median value, which for 82.3% (N=14) of the sample was a week day. Day two was a weekend day for three participants (N=3). This finding links with figure 3.9, where the highest sessional consumption of alcohol was undertaken on just one day.

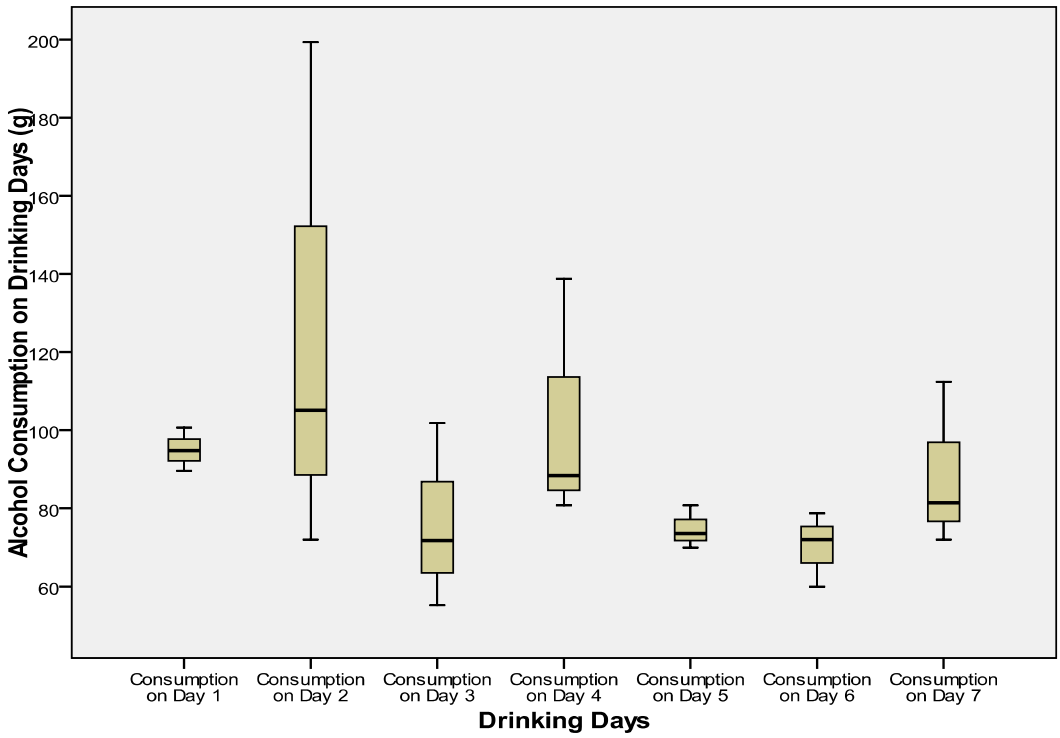

Figure 3.9: Box plots of baseline alcohol consumption (g) per day of diet diary recall within sessional drinkers. *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

Male sessional drinkers consumed alcohol on more drinking days in comparison to females during the baseline week of monitoring. There was a significant difference (p=0.038), in the number of sessional drinking days in a week between male and female sessional drinkers. Males undertook sessional drinking more frequently in the baseline monitoring week in comparison to female sessional drinkers.

3.3.3 ADI Study

The alcohol consumption of the ADI study participants was self-reported by each participant using the study questionnaire. Table 3.6 describes the alcohol consumption within the sample of alcohol-dependent individuals.

Table 3.6: Alcohol consumption by ADI study participants (N=18)

First contact with Alcohol Problems Service (APS) (N)	38.9% (7) <1 year of contact 38.9% (7) 2-10 years of contact 22.2% (4) >10 years of contact
Type of Alcohol Consumed (N)	55.5% (10) consumed one type of alcoholic beverage 44.4% consumed two or more types of alcoholic beverage
Frequency of Consumption in Last Year (N)	83.3% (15) Daily 16.7% (3) Weekly
Days Since Last Alcoholic Drink before Blood Sampling (Median; Mean (SD; Range))	2; 2, (1; 1-7)
Hours Since Last Alcoholic Drink before Blood Sampling (Median; Mean (SD; Range))	48; 50 (37; 24-168)
Reported number of days in month when alcohol was consumed (Median; Mean (SD; Range))	30; 29 (4;15-31)
Alcohol Consumed/Daily (g) (Median; Mean (SD; Range))	222.0; 245.8 (125.4; 80-678.6)

More than half of the sample, 55% (N=10) reported consumption of just one type of alcoholic drink (spirits (N=6), cider (N=2), beer (N=2)). A high percentage of the sample consumed alcohol on a daily basis (median value of 2 alcohol free days before entering detoxification treatment). The grams of alcohol consumed daily before entering treatment encompassed a wide range with the lowest value being 80 g and the highest daily alcohol consumption level being 678.6 g.

3.3.4 Comparison of Alcohol Consumption between Healthy Individuals and Alcohol Dependent Individuals

Alcohol consumption data was gathered from all study participants. At baseline data from the ADI study was compared with data obtained from the HI study and is shown in figure 3.10.

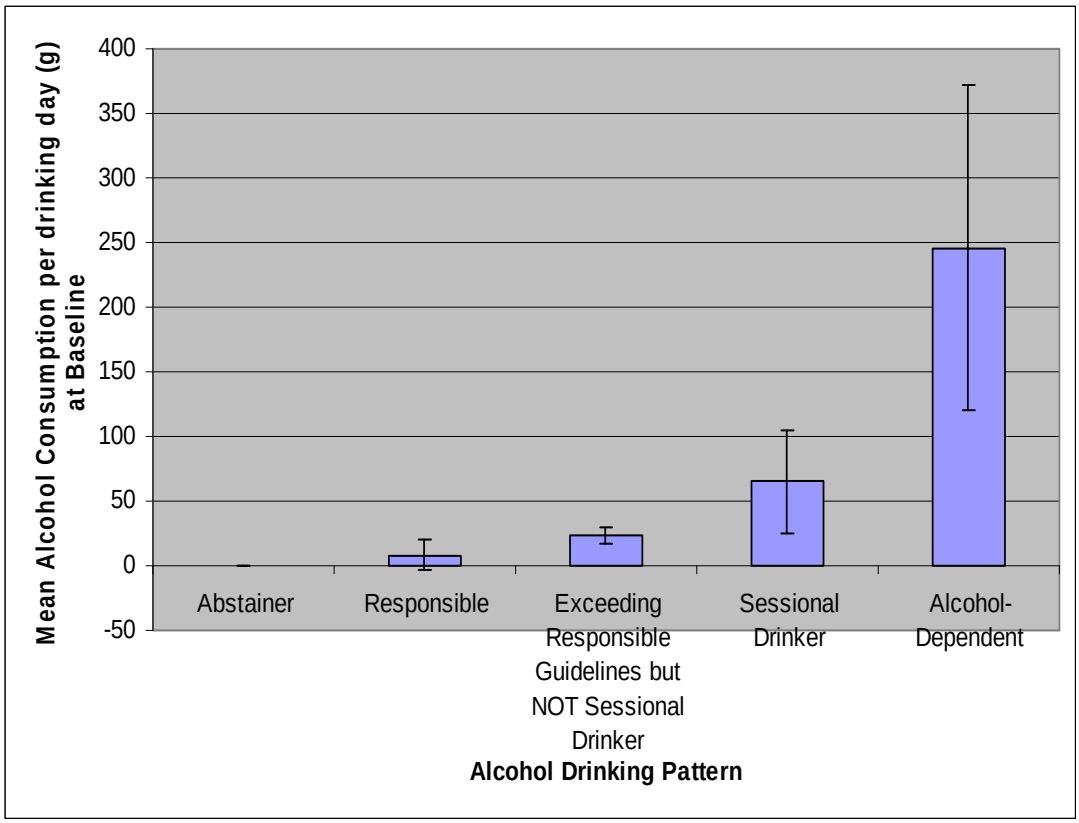

Figure 3.10: Mean alcohol consumption per drinking day of HI study participants (N=35) and ADI study participants (N=18) at baseline. Error bars represent standard deviation (SD).

The direct comparison of the heaviest drinkers in the HI study (sessional drinkers) with alcohol dependent individuals is shown in figure 3.11. The median alcohol consumed by sessional drinkers is lower that that of alcohol dependent patients. There was one outliner in each sample group, who consumed alcohol in an amount out with the range of the total sample. There was a significant difference ($p<0.05$) in the grams of alcohol consumed per day by sessional drinkers when compared to alcohol dependent individuals. However figure 3.11 clearly shows that sessional drinkers can consume the same grams of alcohol per day as an alcohol dependent individual.

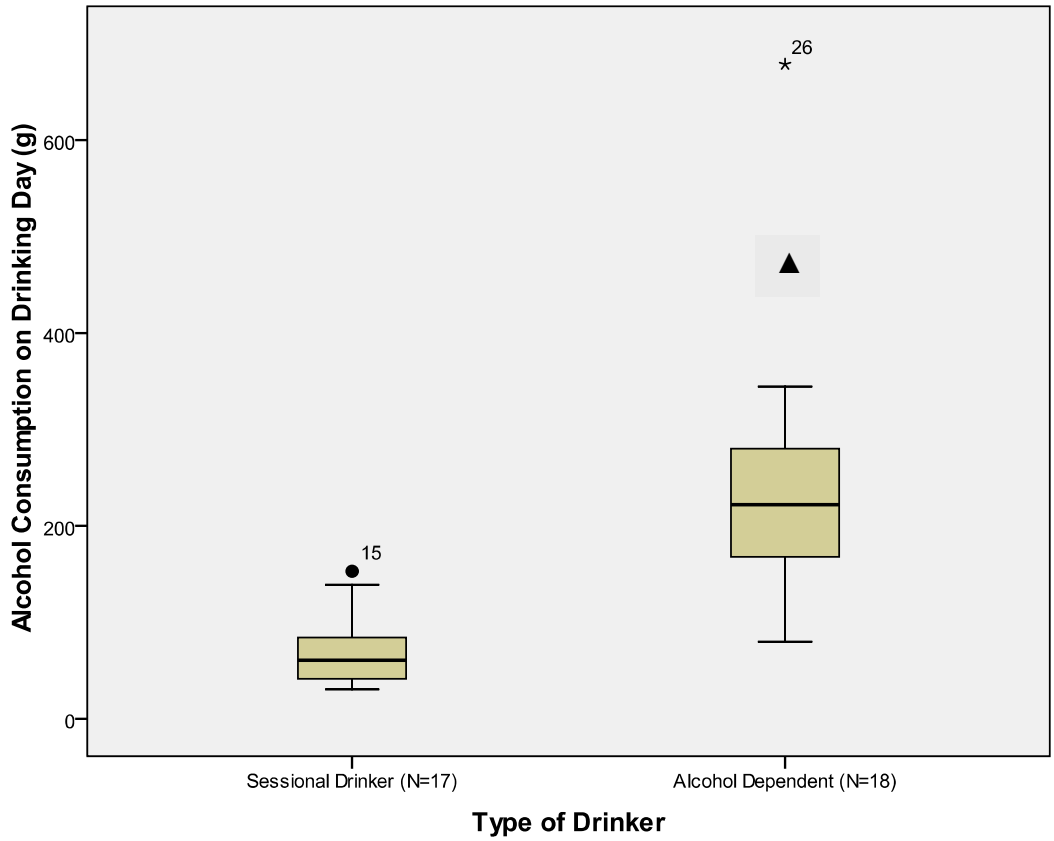

Figure 3.11: Box plot of alcohol consumption (g) per daily of sessional drinkers and alcohol dependent individuals (▲p<0.05, Mann-Whitney test). *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

3.4 Carbohydrate Deficient Transferrin (CDT): HPLC Method Development

This method outlined by Helander et al. 2003 was implemented at QMU and University of Edinburgh laboratories as described in chapter 2. This section will describe the results from the HPLC method development and the resulting use of the N-Latex CDT immunoassay for CDT sample analysis.

3.4.1 Sample Preparation and Mobile Phase Development

The preparation of serum samples for CDT analysis by HPLC involves the complete saturation of iron within the sample and lipid precipitation. This is done by the addition of 20 µl FeNTA solution and 20 µl dextran sulphate-$CaCl_2$. The serum sample prepared as above and using the mobile phase gradient detailed in the method was injected into the HPLC system and the resulting chromatogram is shown in figure 3.12.

The chromatogram in figure 3.12, does show a small peak at the retention time where transferrin should approximately elute from the column, according to the method by Helander et al. (2003). However, the peak is only slightly visible. To improve the separation of the transferrin glycoforms on the chromatogram, the volumes of FeNTA and dextran sulphate-CaCl2 were increased to 40 µl. The gradient of the salt concentration within the mobile phase was also altered to increase the volume of salt being pumped into the column. The gradient was increased to 100%, whereby the mobile phase buffers were not increased on a gradient incline, but were at the same flow rate, throughout the duration of the HPLC run. The result of this alteration to the mobile phase and sample preparation is shown in chromatogram figure 3.13.

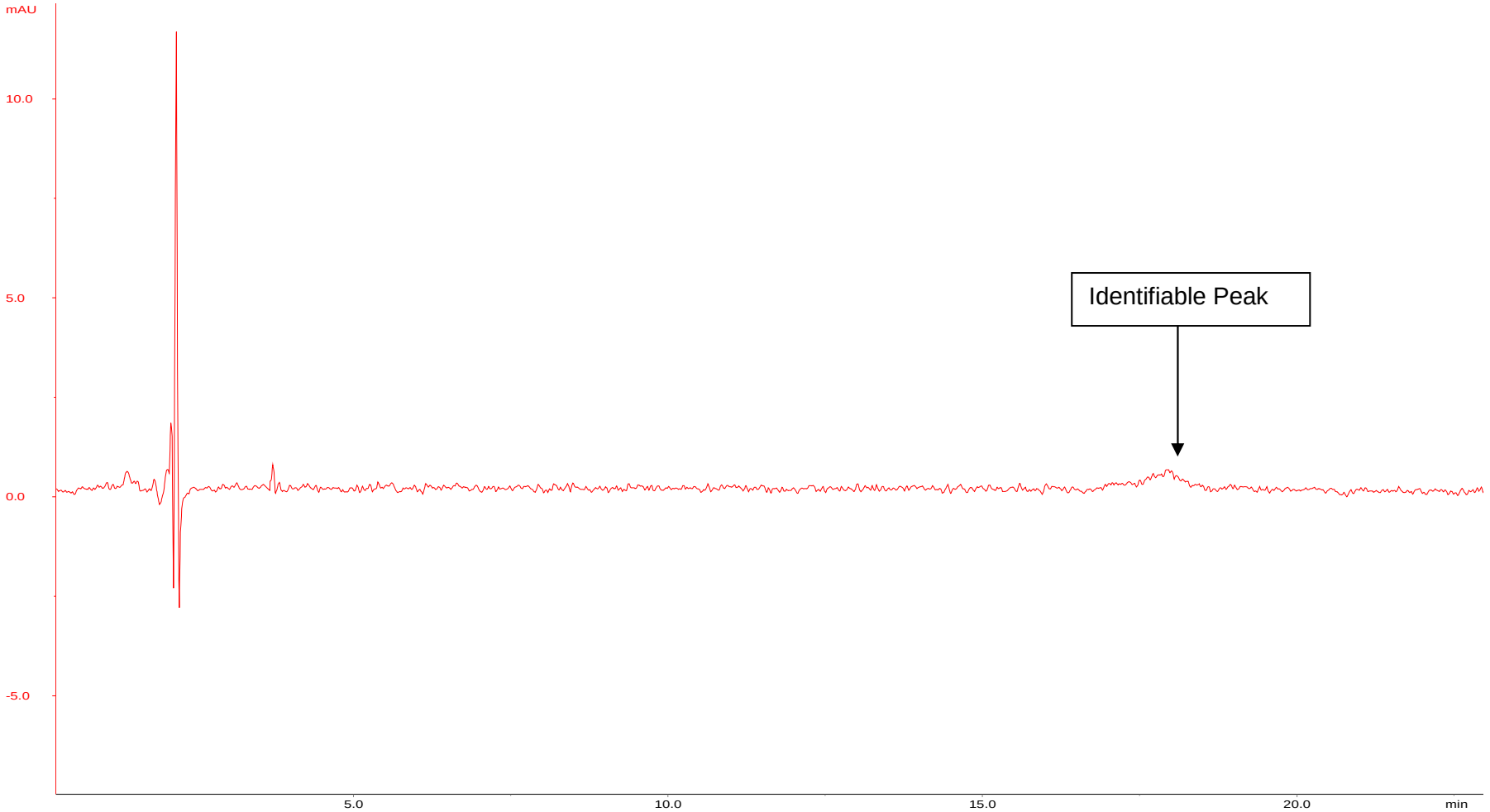

Figure 3.12: 20% Salt Gradient Mobile Phase and 20 µl FeNTA and Dextran Sulphate-$CaCl_2$. (X-axis = retention time (minutes); Y-axis = absorbance (mAU). Detector: ultraviolet (UV); volume injected: 400 µl; wavelength: 470 nm; total run time: 20 minutes.

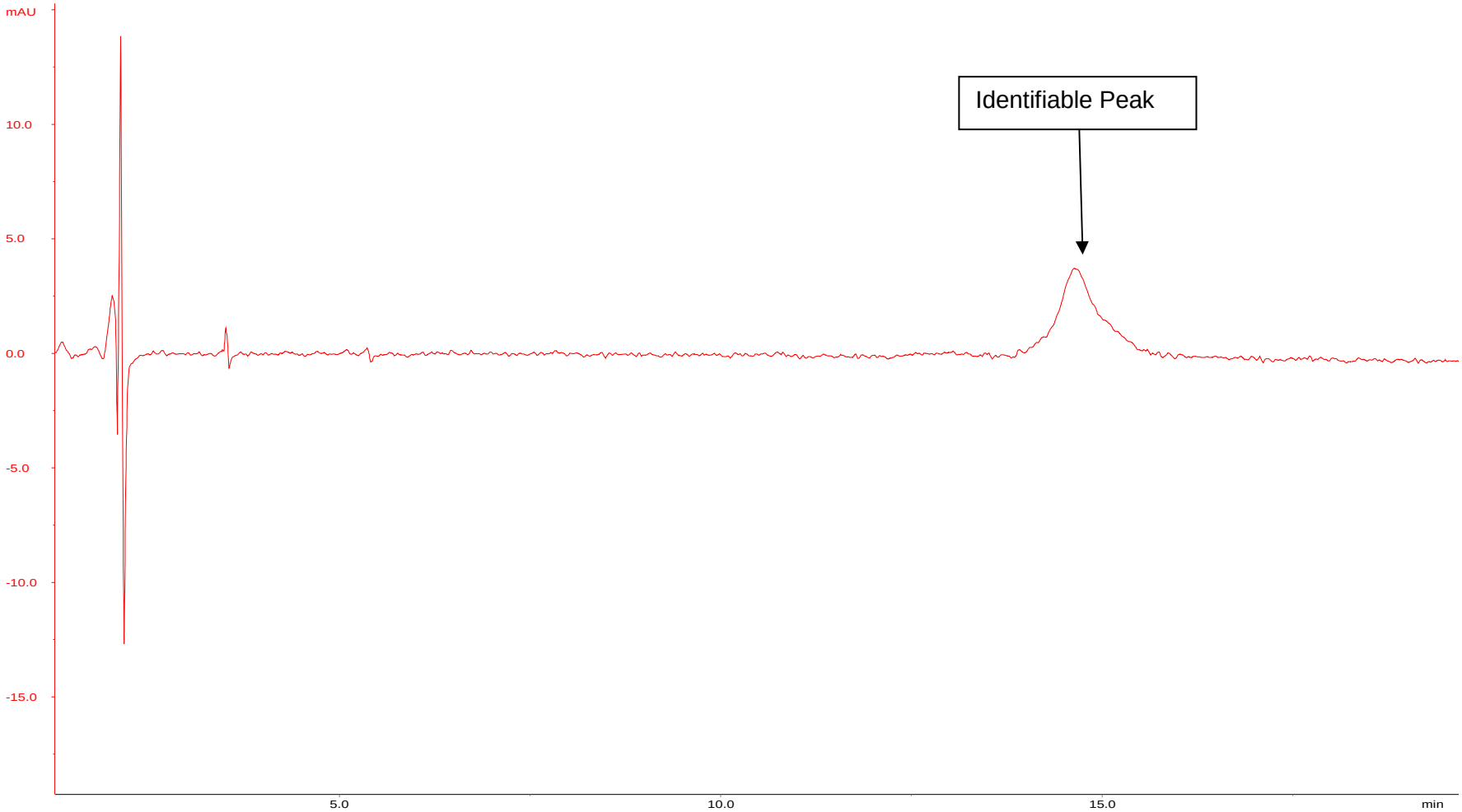

Figure 3.13: 100% salt gradient mobile phase and 40 µl FeNTA and Dextran Sulphate-$CaCl_{2.}$ (X-axis=retention time (minutes), Y-axis=absorbance (mAU). Detector: ultraviolet (UV); volume injected: 400 µl; wavelength: 470 nm; total run time: 20 minutes.

As illustrated in the chromatogram (figure 3.13), the increased salt mobile phase gradient, produced an increased iron saturation and lipid precipitation which provided a clearer and identifiable peak at 15 minutes. As described by Helander et al. (2003) the first transferrin glycoform elutes from the column at approximately 15 minutes. To identify the peak which eluted at 15 minutes, fractions were collected and subjected to protein gel electrophoresis and mass spectroscopy, as described in chapter 2.

3.4.2 Protein Gel Electrophoresis

Ten fractions were collected from the HPLC run which identified a protein peak at 15 minutes. The fractions collected were subjected to protein gel electrophoresis to identify if a CDT glycoform had eluted from the column. Figure 3.14 shows the gel electrophoresis result and highlights the presence of a protein which has a similar weight to the glycoforms which form CDT. This suggested that further work was required to identify which CDT glycoforms were eluted from the HPLC column at 15 minutes.

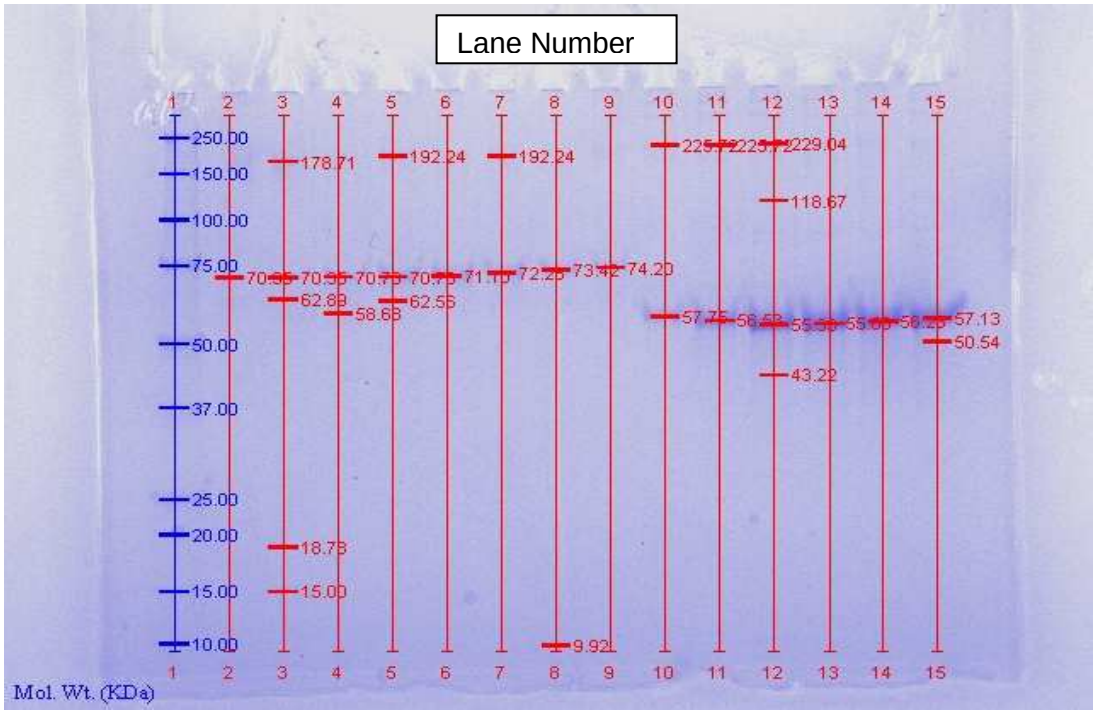

Figure 3.14: Protein gel electrophoresis of fractions collected from the HPLC analysis shown in figure 3.13.

Mass spectroscopy analysis was carried out on the fractions to further identify if one or all CDT glycoforms were present. From the literature the molecular weights of the transferrin glycoforms which form CDT were identified and are shown in table 3.7.

Table 3.7: Molecular Weight of CDT Glycoforms (Oberrauch, Bergman and Helander 2008; Peter et al. 1998)

CDT Glycoform	Molecular Weight (Da)
Asialotransferrin	75157
Monosialotransferrin	77180
Disialotransferrin	77365
Triaialotransferrin	79281
Tetrasialotransferrin	79573
Pentasialotransferrin	80232/80379

Transferrin glycoforms, which form CDT, were detected in each of the fractions collected, which was confirmed by mass spectrometry analysis. The primary analyte for CDT analysis, Disialotransferrin (Jeppsson et al. 2007), was confirmed as being present in the fractions collected from the HPLC analysis, as illustrated in figure 3.15.

From the data generated from the mass spectroscopy (figure 3.15), it can be suggested that within the peak which eluted at 15 minutes during the HPLC run there was evidence of the presence of CDT glycoforms, as the presence of the corresponding molecular weights were present in the mass spectroscopy spectrums. However more work is required to fully investigate the exact contents of the peak which eluted at 15 minutes.

3.4.3 Summary

After subsequent method development using various techniques, the CDT HPLC method for the detection of CDT in human serum could not be validated at either QMU or University of Edinburgh laboratories. At both laboratories the separation of the CDT glycoforms using HPLC was not successful, meaning the concentration of

the CDT glycoform which is affected by alcohol consumption, namely Disialotransferrin could not be determined. This was due to the inability to separate the individual glycoforms which form CDT. Further work is needed to validate this HPLC method. It was for these reasons that, the N-Latex immunoassay method for CDT analysis implemented at the Northern General Hospital in Sheffield was used to analyse selected serum samples for CDT from the HI, ADI and stability studies.

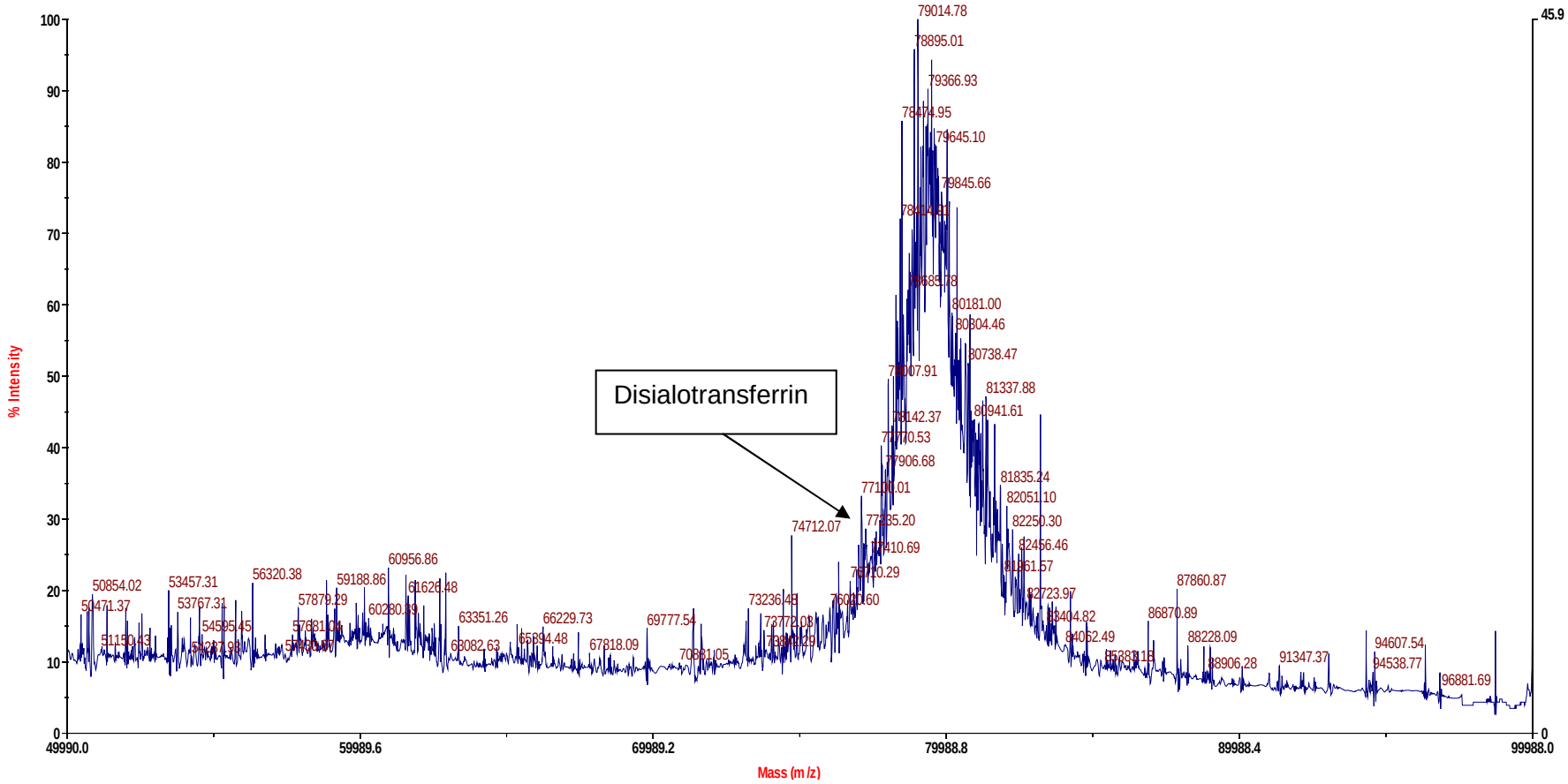

Figure 3.15: Mass spectroscopy of fraction E9, showing presence of CDT glycoform, Disialotransferrin. (Sample Volume: 0.5 µl; Matrix: Sinapinic acid; Matrix Volume: 0.5 µl; Total Well Volume: 1 µl; Polarity: Positive; Laser intensity: 2086; Accelerating Voltage: 25,000 volts).

3.5 Carbohydrate Deficient Transferrin (CDT) and Alcohol Consumption

The consumption of alcohol was determined using questionnaires and diaries. Alcohol consumption was also determined using biological biomarkers of alcohol consumption, which reduces the errors associated with memory recall and accuracy in recording alcohol consumption.

3.5.1 CDT Levels within Healthy Individuals (HI) who Consume Alcohol in a Range of Patterns

CDT was measured in serum using the N-Latex immunoassay at the Northern General Hospital in Sheffield, as described in chapter 2. A positive test result using the CDT N-Latex immunoassay is indicated by a result of >2.6% of bound CDT in the serum sample. A participant is deemed to have a positive test result for CDT levels, if the result is greater than 2.6% of bound CDT in the serum sample analysed. If the result is greater than 2.6%, this result is indicative of the participant drinking in a harmful pattern and above the responsible UK drinking guidelines. The percentage of CDT identified in each of the alcohol drinking pattern groups is presented in figure 3.16. There was no significant difference in the levels of serum %CDT found in each alcohol drinking group at each of the pre-defined study time-points, illustrated in figure 3.16.

CDT is calculated by according to the instructions and calibrators provided within the Dade Behring N-Latex kit. A reference curve is generated by multi-point calibration, which is performed by the Dade Behring BN ProSpec© System. The results of each sample batch are performed automatically and the calculation of %CDT is integrated within the software used by the Dade Behring BN ProSpec© System. The principle of the N-Latex method and calculation of %CDT is explained fully in chapter 2, section 2.4.2.2.

Due to the high running costs of sample analysis by the N-Latex immunoassay, only 12 study participants' samples were analysed. Study participants were selected from baseline alcohol consumption data, whereby serum samples analysed were from participants who either abstained from alcohol (control) or consumed alcohol

according to the definition of sessional drinking. Serum samples from the selected study participants were analysed for every study time-point.

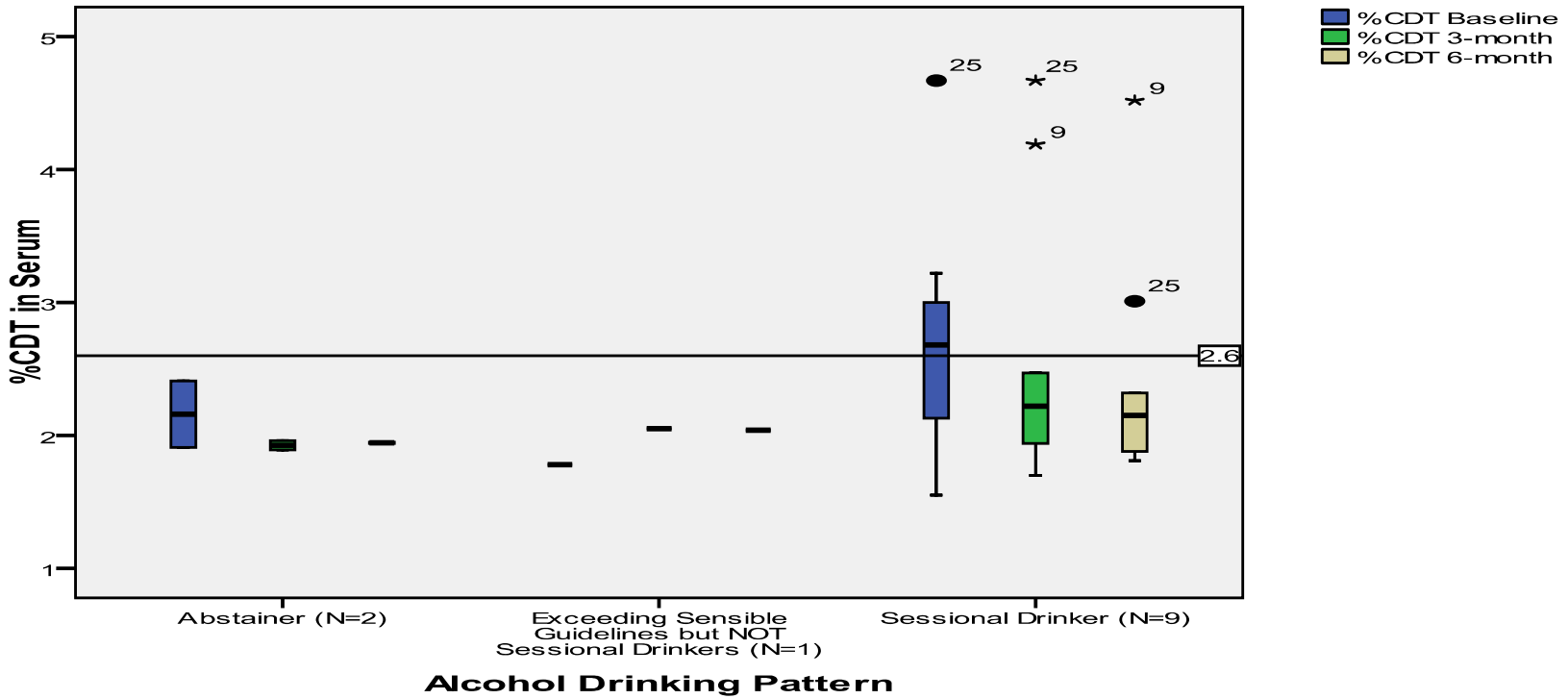

Figure 3.16: Box plots of %CDT in serum of HI study (N=12) sample categorised according to alcohol drinking pattern as determined by the AM method from diaries at baseline. Horizontal line represents the cut-off value for a positive CDT result of >2.6%. *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score).*

*● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box).*

%CDT at baseline and mean alcohol consumption during drinking days at baseline was not significant (p=0.656, R=-0.144), for all HI study participants who were selected for CDT analysis. This association between the two variables was also not significant at the 3-month time-point. However at the 6-month time-point, a significant positive correlation was identified between %CDT and mean alcohol consumption during drinking days (p=0.027, R=0.635). The association, at the 6-month time-point in shown in figure 3.17.

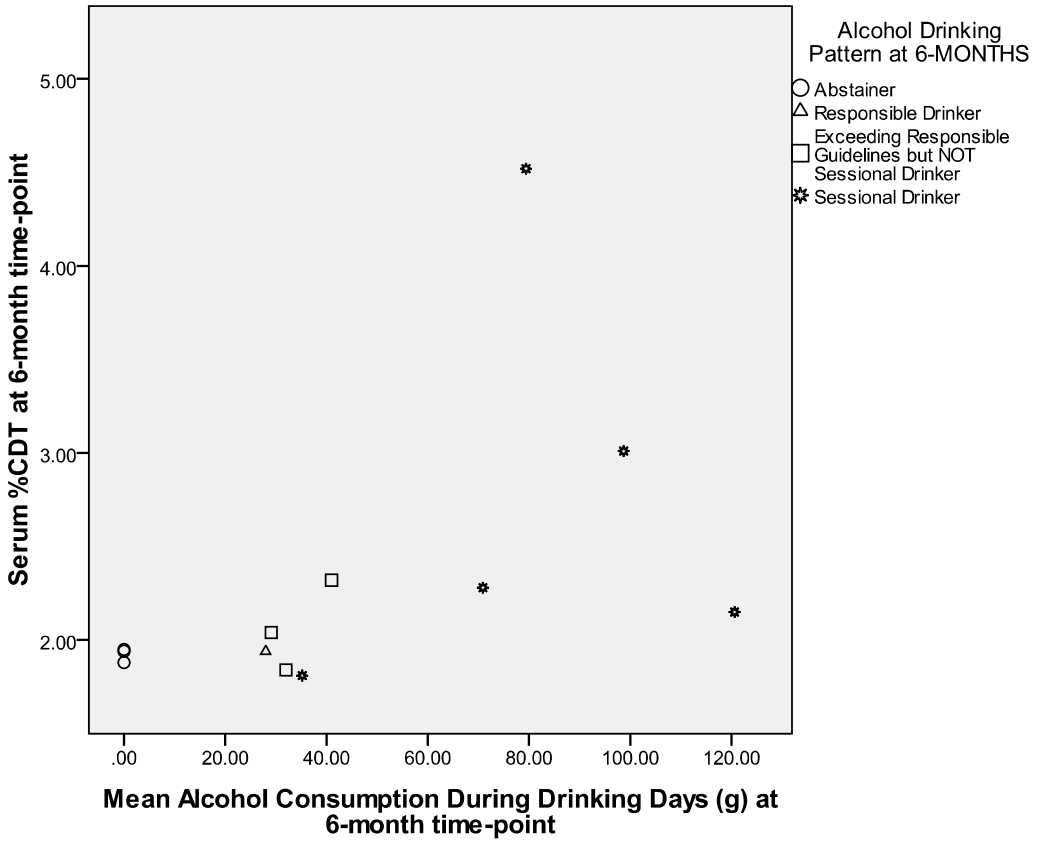

Figure 3.17: Scatter plot of % CDT at baseline in serum for HI study sample (N=12) in relation to 6-month mean alcohol consumption per drinking day in grams, from diary (AM method) (p=0.027, R=0.635 Spearman's test).

Of the HI study sample tested for CDT levels (N=12), 41.7% (N=5) had a positive test result of greater than 2.6% using the N-Latex immunoassay. All were categorised from diaries as sessional drinkers.

3.5.2 CDT Levels within Alcohol Dependent Individuals (ADI)

The percentage of CDT (%CDT) identified in the ADI study sample at predetermined study time-points is shown in figure 3.18.

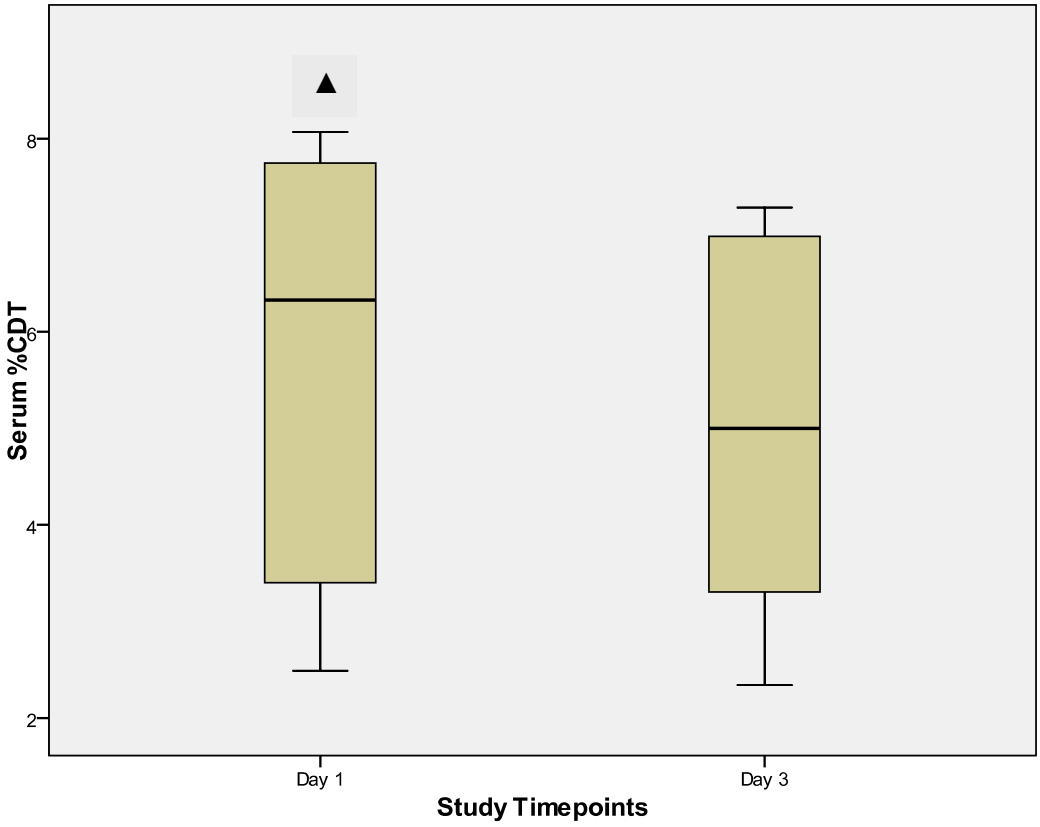

Figure 3.18: Box plots of %CDT in serum in ADI study sample (N=18) at two predetermined study time-points (▲p=0.017, Mann-Whitney Test). *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

The box plots shown in figure 3.18 highlight the significant reduction (p=0.017) in CDT levels from day 1 to day 3, which has been influenced by alcohol detoxification treatment. A dot plot, as shown in figure 3.19, shows that 69.2% (N=13) of the study sample had a positive %CDT result on day 1.

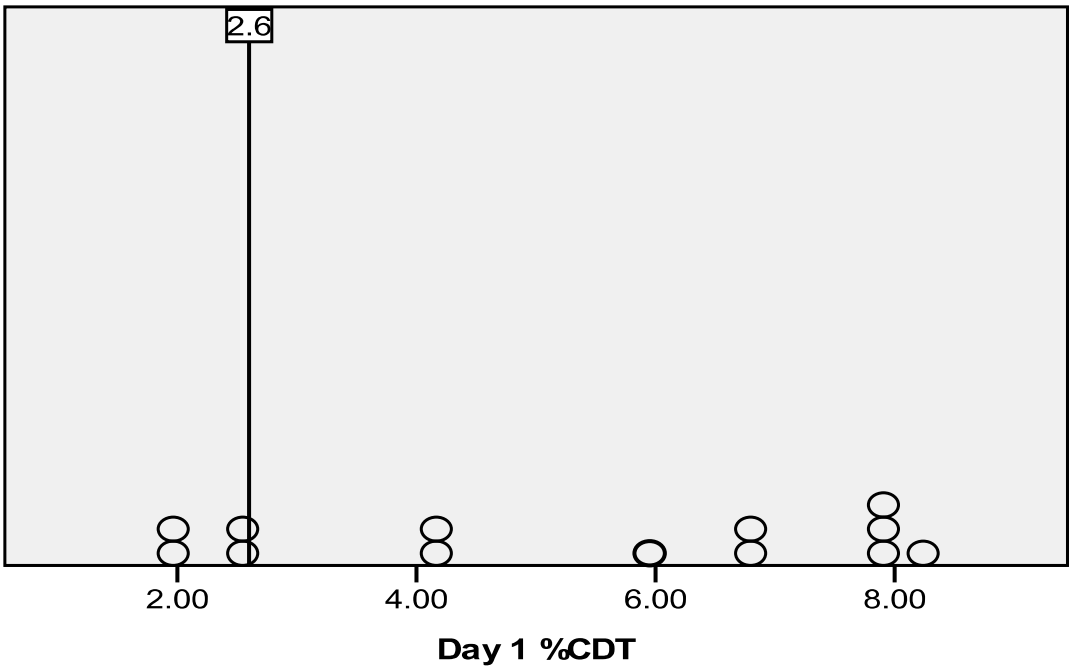

Figure 3.19: Dot plot %CDT in serum of ADI participants (N=13) on day 1, in relation to positive result. The vertical reference line represents the reference cut off value of >2.6% as a positive result within the %CDT N LATEX assay.

3.5.3 CDT Levels within Drinkers Combined Sample (HI and ADI)

In the sample of all drinkers (N=23), regardless of drinking pattern, the range of %CDT on day 1 was 1.55%-8.09%. The sample number for CDT analysis decreased from N=44 to N=23, due to the limited provision for CDT analysis.

The relationship between alcohol consumption and %CDT is shown in figure 3.20. An association between alcohol consumption and %CDT on day one/baseline was not significant (p=0.123, R=0.331). The R value was recalculated after removal of the outlier (alcohol consumption of >600 g), which produced a significant correlation

(p=0.027, R=0.469). The participant, who recorded in their study questionnaire that they consumed greater than 600 g of alcohol prior to alcohol detoxification treatment, was found to have a negative %CDT result, which would not be consistent with the volume of self-reported alcohol consumption. Literature evidence (Jeppsson et al. 2007) suggests that the half-life of CDT is 2 weeks, which could indicate that this individual, could have consumed approximately 600 g of alcohol, however this was not within the 2 week period prior to blood sampling, further warranting the removal from correlation analysis. This finding suggests that the participant was not accurate in his questionnaire completion. This suggests that CDT can be used as a biomarker in a sample of individuals who consume alcohol in range of patterns, including non-dependent patterns.

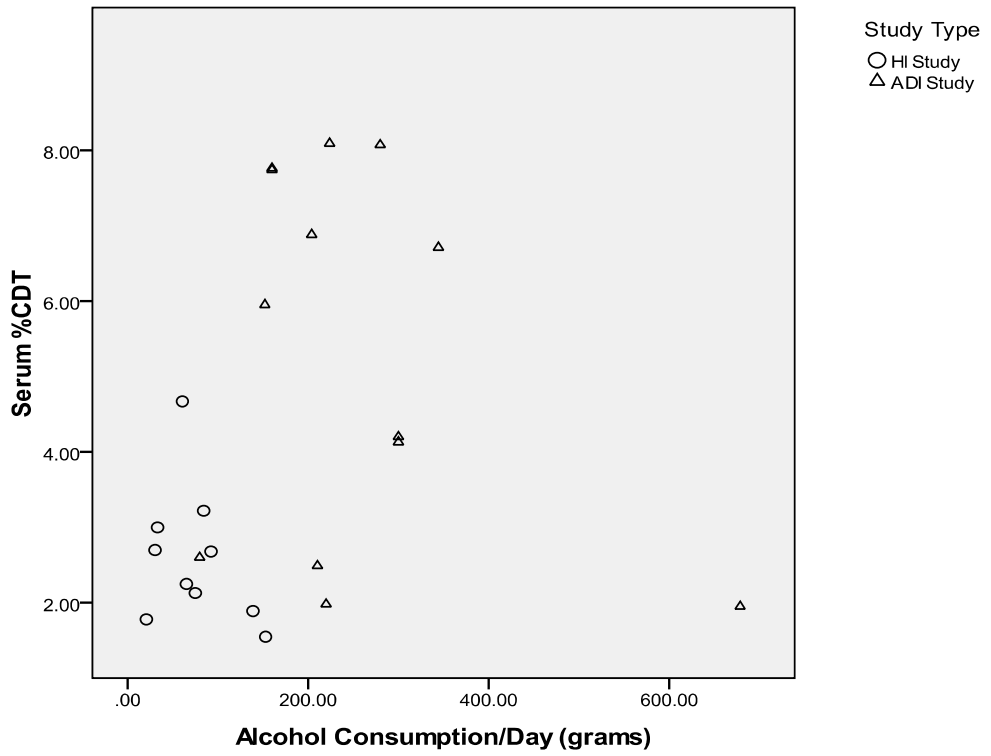

Figure 3.20: Serum %CDT plotted as a function of alcohol consumption per day. (p=0.027, R=0.469, Spearman's test).

3.5.4 CDT Levels in a Sample of Sessional Drinkers

Literature suggests that serum CDT can be elevated following the consumption of more than 50 g of alcohol daily over a consistent period of 2 weeks (Jeppsson et al. 2007). The %CDT cut off value for a positive result is shown in figure 3.22, where

five sessional drinkers, within the study sample have a positive CDT result of greater than 2.6%. This is indicative of 55.6% of the total sample of HI sessional drinkers (N=9), tested for CDT levels, having a positive result, indicating harmful drinking.

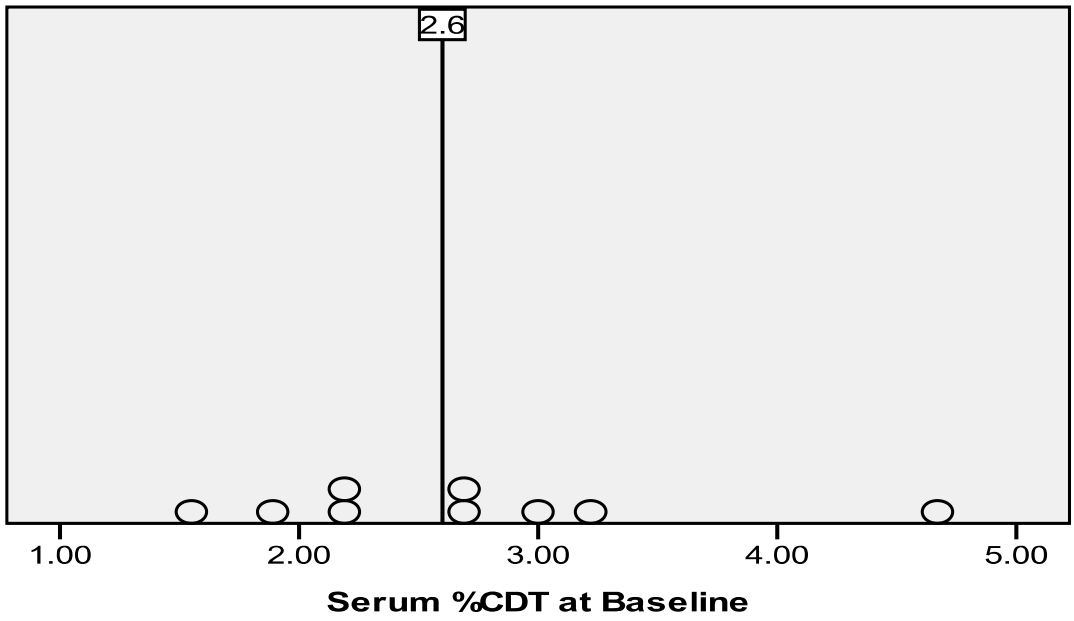

Figure 3.21: Dot plot of %CDT in serum within sessional drinker's sample (N=9), in relation to positive test result. The vertical reference line represents the reference cut off of >2.6% as a positive result within the %CDT N LATEX assay.

The value of %CDT at baseline did not significantly correlate with the number of drinking days within a week, using a Spearman's test (p=0.124, R=0.551), which was also found at the three and six month time-points, in a sample of sessional drinkers. A significant linear relationship (p=0.01, R=0.98) between the number of drinking days and the mean %CDT at baseline within a sample of sessional drinkers was identified (figure 3.22).

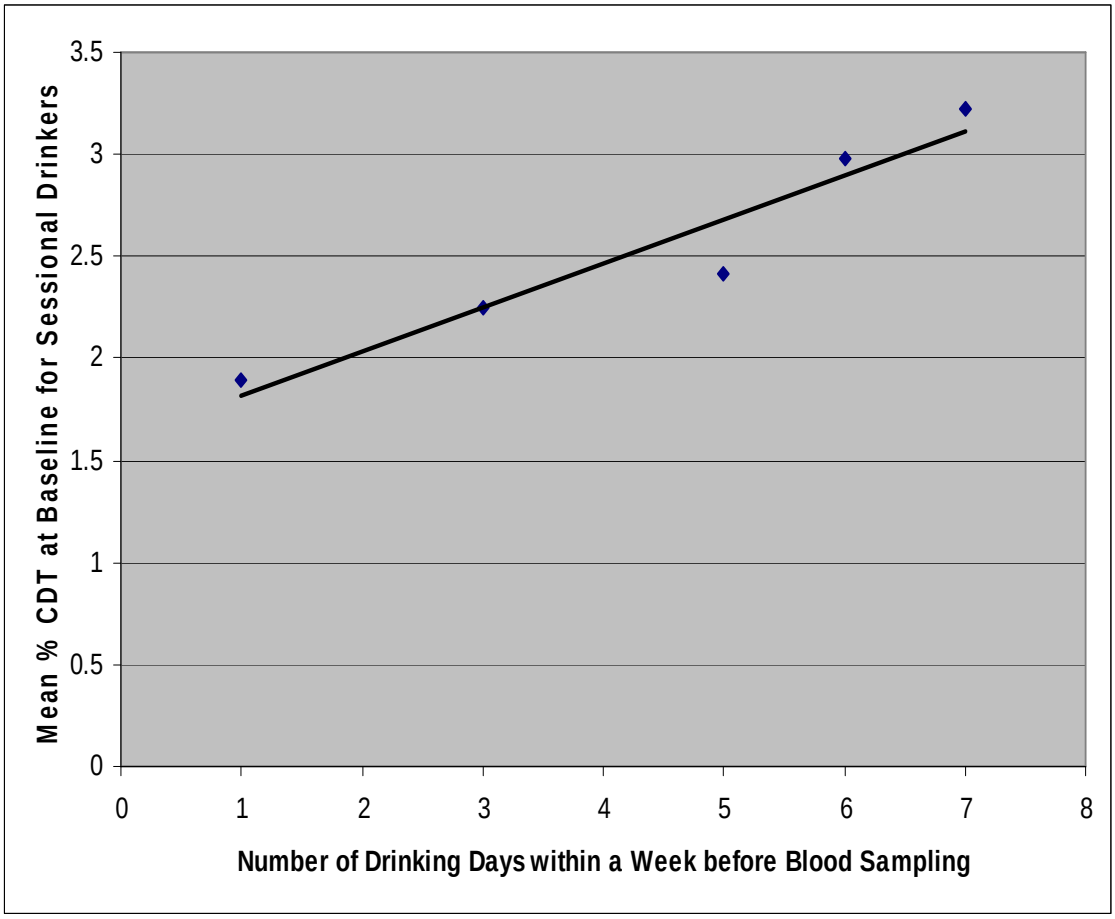

Figure 3.22: Linear relationship between %CDT at baseline in sessional drinkers (N=9) and number of drinking days in a week (p=0.01, R=0.98 Spearman's test)

3.5.5 CDT Levels Compared in Samples of Sessional Drinkers and Alcohol Dependent Individuals

The median levels of serum %CDT were higher in alcohol dependent patients in comparison to sessional drinkers; as illustrated in figure 3.23. There was one outlier within the sessional drinkers group, which was above the box plot range. A significant difference between %CDT levels in sessional drinkers and alcohol dependent patients (p=0.030) was evident. This result suggests that CDT levels are significantly different between the two studies; however the median %CDT levels for both studies were above the 2.6% cut-off value for a positive result, suggesting that sessional patterns of alcohol consumption and alcohol-dependent consumption can affect CDT levels.

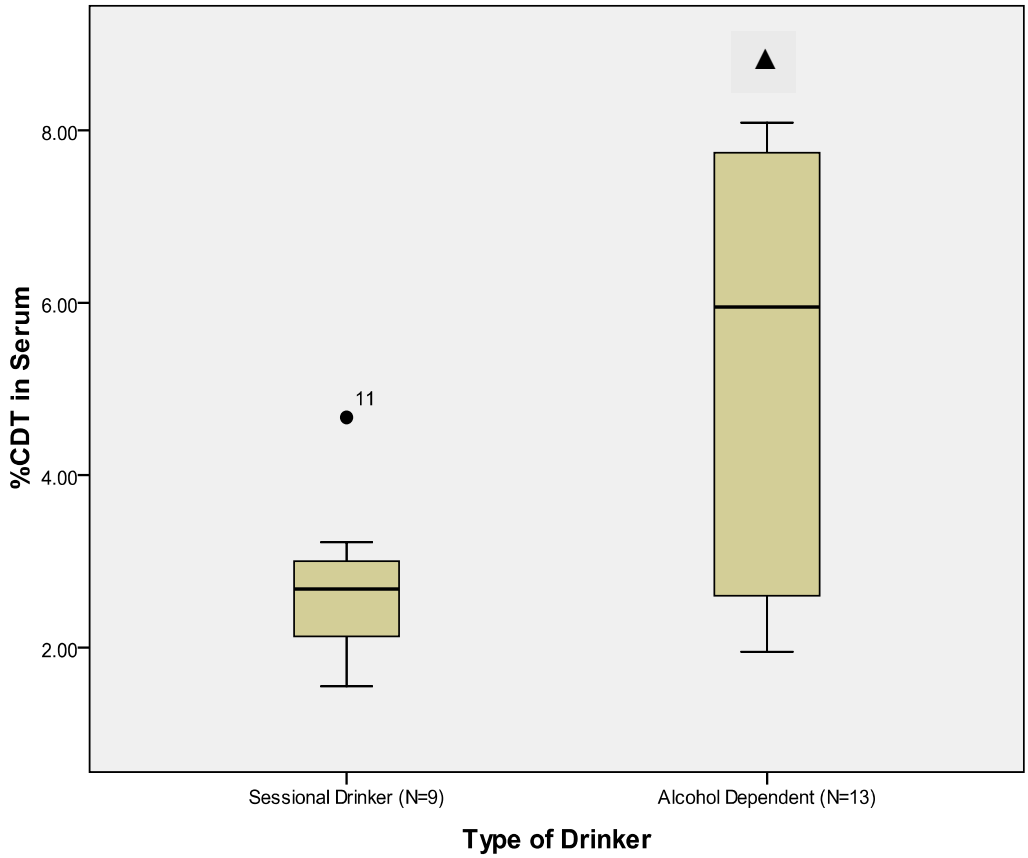

Figure 3.23: Box plots of serum %CDT in a sample of sessional drinkers compared to alcohol dependent patients (▲p=0.030, Mann-Whitney test) *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

3.6. Validation of Plasma Homocysteine Assay

3.6.1 Linearity

The calibration graph for the determination of homocysteine in plasma is shown in figure 3.24. The calibration graph of an aqueous solution of homocysteine was linear in the concentration range of 0-20 µmol/l. The equation of the regression graph was y=33258X+76144. The coefficient of determination for the calibration curve, R^2=0.998, which is greater than 0.99 which is the value required for an

accurate HPLC analysis. A sample chromatogram of a homocysteine standard is shown in figure 3.25. Furthermore figure 3.26 shows a chromatogram of homocysteine detected in a plasma sample.

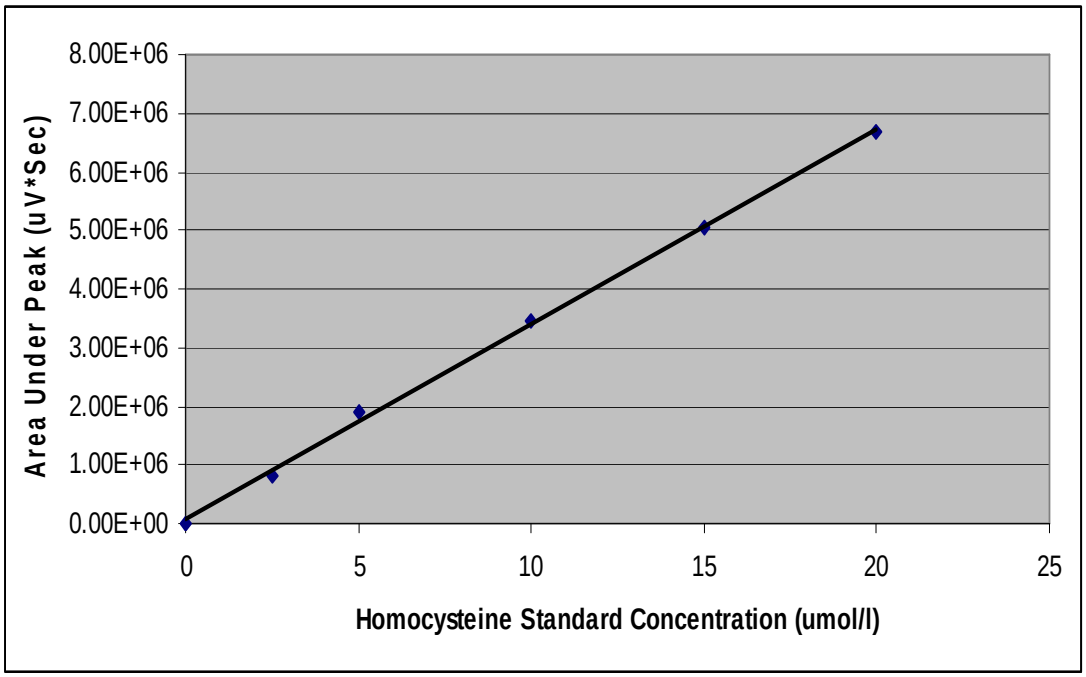

Figure 3.24: Area under the peak plotted as a function of homocysteine standard concentration (µmol/l). (R=0.9994)

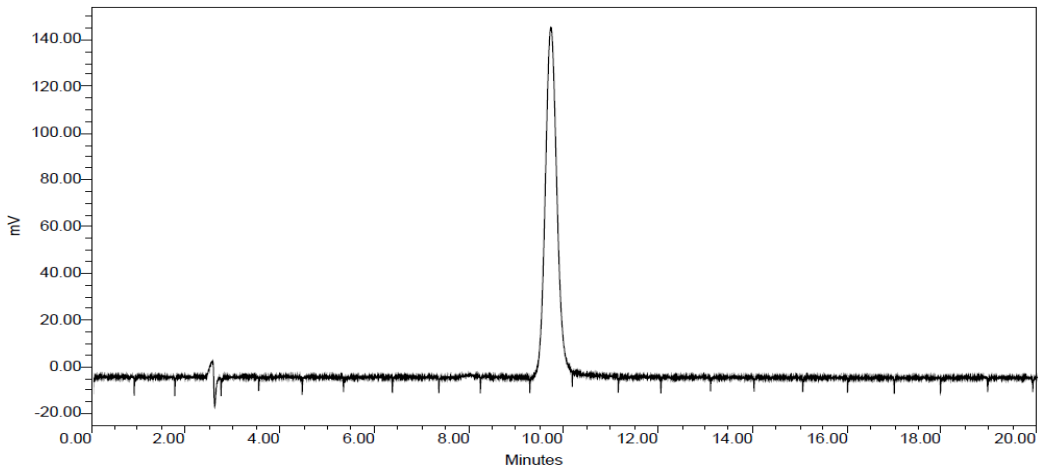

Figure 3.25: Chromatogram showing a homocysteine standard separation by HPLC. MilliVolts (mV) plotted as a function of time (minutes). (Detector: electrochemical (ED); Volume Injected: 20 µl; Sensitivity Setting: +0.86 volts; Flow Rate: 0.8 ml/minute; Total Run Time: 20 minutes).

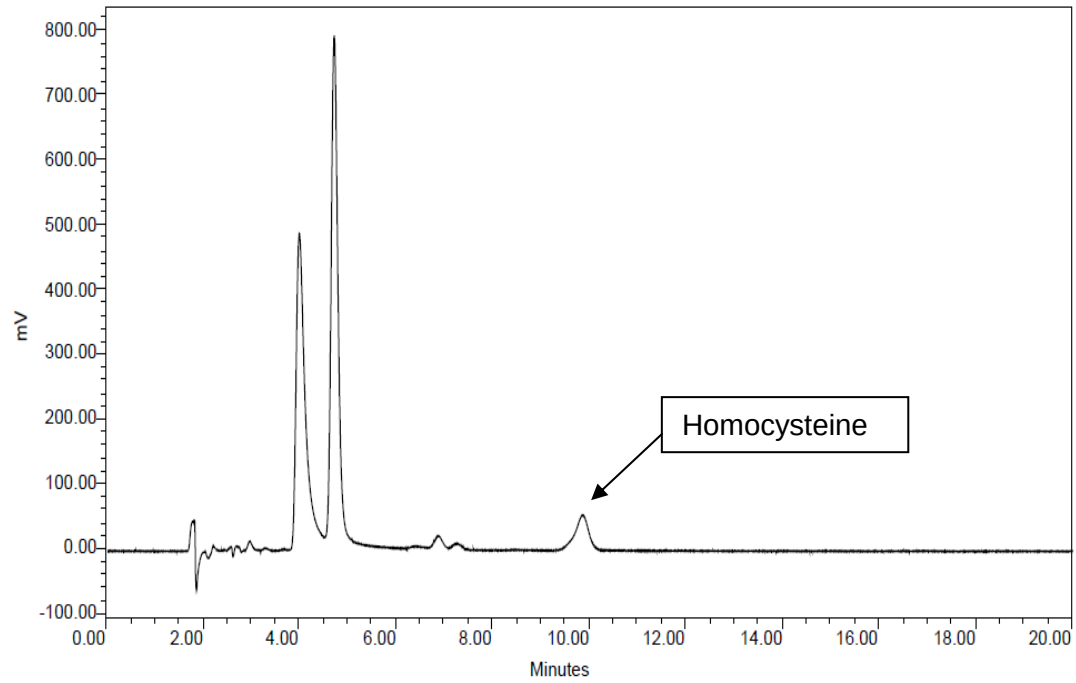

Figure 3.26: Chromatogram showing homocysteine in plasma separated using HPLC. MilliVolts (mV) plotted as a function of time (minutes). (Detector: electrochemical; volume Injected: 20 µl; sensitivity setting: +0.86 volts; flow rate: 0.8 ml/minute; total run time: 20 minutes).

3.6.2 Precision (Intra and Inter-assay)

Duplicate injections of the homocysteine standards 25% and 200% (which represents the upper and lower concentrations of the range) were performed. The %CV was compared for HPLC runs conducted on the same day (intra-assay) and compared with results obtained on different days (inter-assay) as shown in table 3.8 and 3.9 respectively.

Table 3.8: Intra-Assay measurements for both 25% and 200% concentrations of homocysteine.

Standard Concentration (%)	Injection 1 % (µmol/l)	Injection 2 % (µmol/l)	Injection 3 % (µmol/l)	Mean ±SD % (µmol/l)	CV (%)
200% (20 µmol/l)	195.2 (20.6)	195.2 (20.6)	195.2 (20.6)	195.2±0.0 (20.6±0.0)	0.0 (0.0)
25% (2.5 µmol/l)	19.1 (2.0)	18.7 (1.9)	18.9 (2.0)	18.9±0.2 (2.0±0.00)	1.0 (0.0)

Table 3.9: Inter-Assay measurements for both 25% and 200% concentrations of homocysteine.

Standard Concentration (%)	Injection 1 % (µmol/l)	Injection 2 % (µmol/l)	Injection 3 % (µmol/l)	Mean ±SD % (µmol/l)	CV (%)
200% (20 µmol/l)	195.5 (20.8)	194.7 (20.6)	194.6 (20.6)	195.3±0.9 (20.6±0.1)	0.4 (0.6)
25% (2.5 µmol/l)	24.9 (2.6)	24.2 (2.6)	24.3 (2.6)	24.5±0.4 (2.6±0.0)	1.6 (1.6)

3.6.3 Summary

The %CV for both intra and inter assays was less than 5%, concluding that this HPLC method is a valid and reliable assay for the detection of homocysteine in plasma.

3.7 Plasma Homocysteine and Alcohol Consumption

Homocysteine was measured in plasma using high pressure liquid chromatograph (HPLC) with electrochemical detection (ED) as described in chapter 2.

3.7.1 Plasma Homocysteine Levels within Healthy Individuals (HI) who Consumed Alcohol in a Range of Patterns

The homocysteine concentration identified in each of the alcohol drinking patterns is represented in figure 3.27.

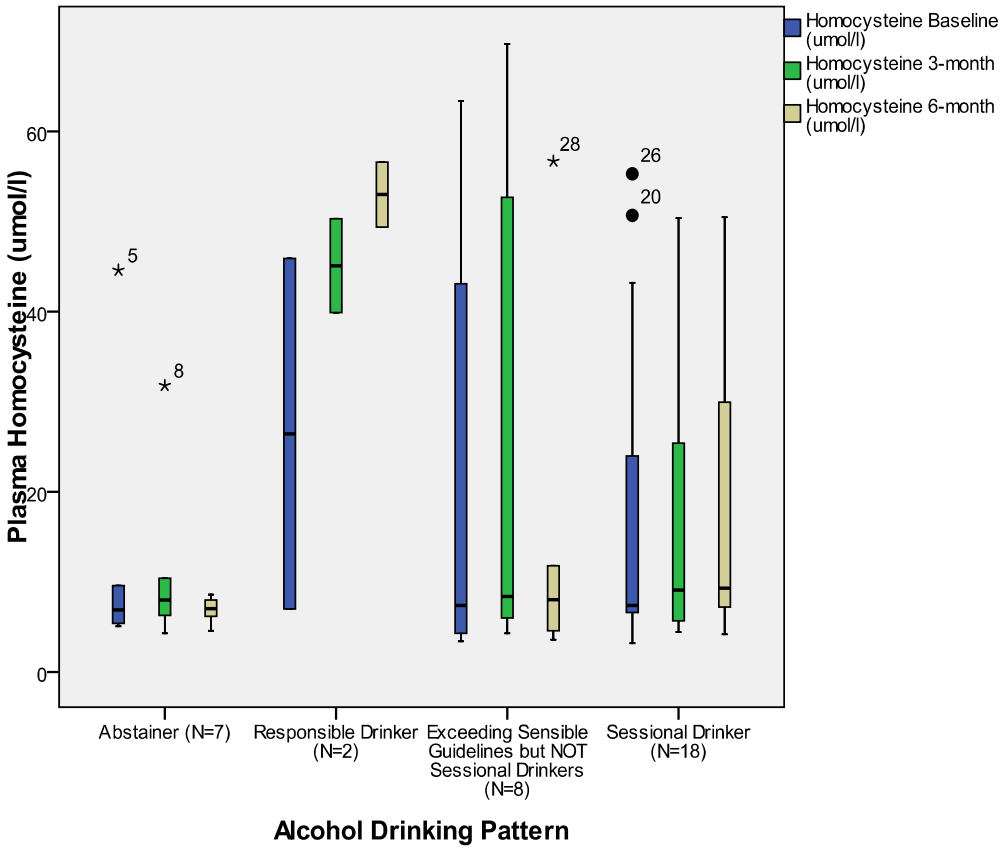

Figure 3.27: Box plots of plasma homocysteine levels (µmol/l) at each study time-point in healthy individuals (HI; N=35) grouped according to alcohol drinking pattern *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

The median plasma homocysteine remained relatively constant and did not change significantly for the total HI study sample over the study period. There was a non-significant increase (p=0.135) in plasma homocysteine levels at each pre-defined study time-point for the responsible drinkers (N=2). There was also no significant difference between plasma homocysteine at each study time-point for the other drinking pattern groups. There was no significant association between baseline plasma homocysteine measurement and number of drinking days at baseline. No significant difference between plasma homocysteine at baseline and frequency of alcohol consumption as stated by participants in the study questionnaire was evident. Plasma homocysteine baseline values and mean alcohol consumption during drinking days at baseline showed no correlation for each of the alcohol drinking patterns (figure 3.28). The association between these two variables was also not significant at the 3-month and 6-month time-points.

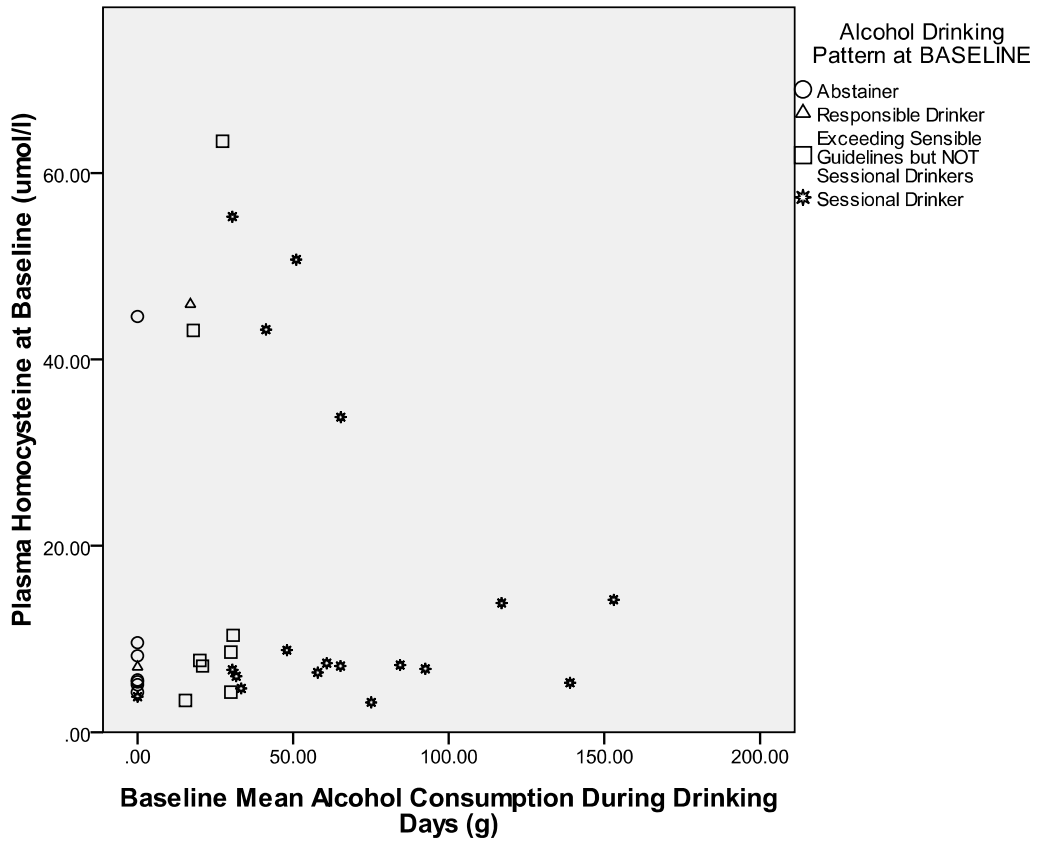

Figure 3.28: Scatter plot of plasma homocysteine concentration (µmol/l) in relation to HI study participant's mean alcohol consumption during drinking days

Plasma homocysteine can be affected by age and gender (Antoniades et al. 2009). However no association between plasma homocysteine baseline measurement and age of participants was evident. There was no significant difference between plasma homocysteine levels in female and male participants at baseline (p=0.099), suggesting there was no difference in the levels of plasma homocysteine between the two genders. There was also no significant difference in plasma homocysteine between the two genders at the three and six month time-points. Figure 3.29, illustrated the plasma homocysteine levels in female and male participants recruited to the HI study, at baseline.

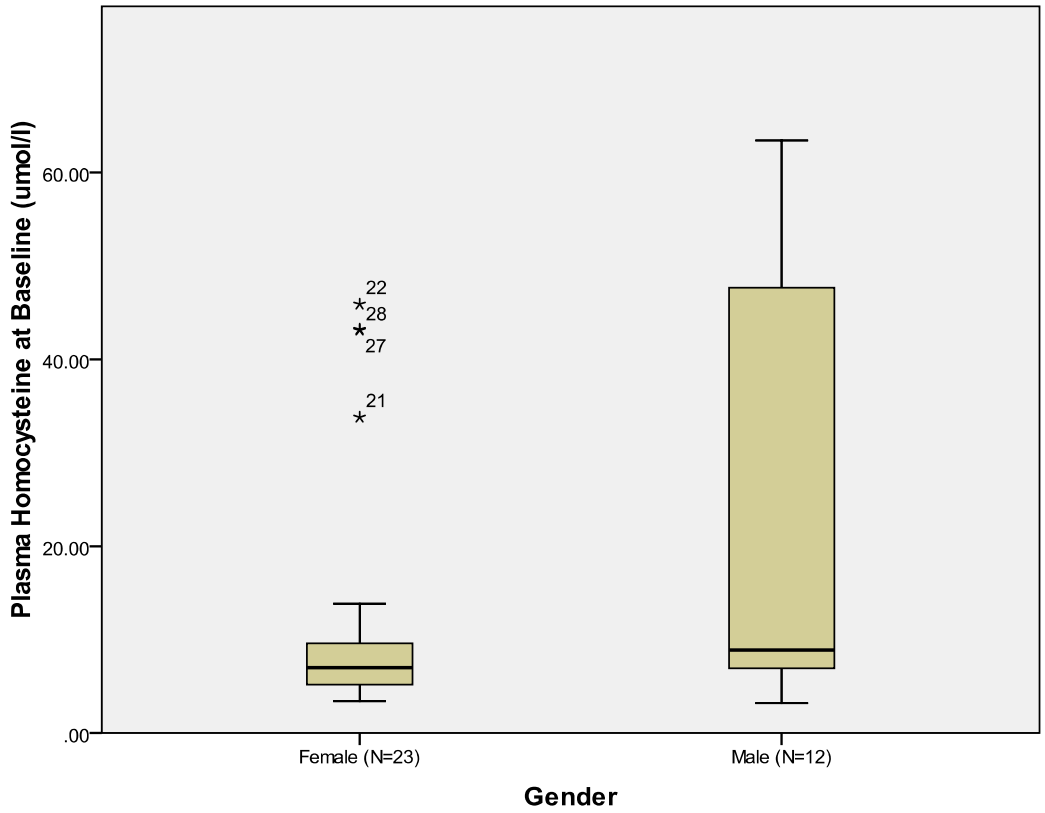

Figure 3.29: Boxplot of plasma homocysteine (µmol/l) at baseline in relation to HI study participant's gender *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box*.

Literature evidence suggests certain lifestyle factors including smoking, hormonal contraception and the menopause can influence plasma homocysteine levels

(Russo et al. 2008). Participants were asked to self-report on their study questionnaire if they were cigarette smokers. There was no significant difference in plasma homocysteine levels at all time-points between smokers (N=4) and non-smokers (N=31), indicating that in the study sample smoking did not have an effect on plasma homocysteine levels. Female participants were asked to state if they used hormonal contraception and if they were menopausal. No female participants were recruited into the HI study that were menopausal. The participants who used hormonal contraception (N=9), were asked to state the brand of hormonal contraception used, either combined (N=5) or progesterone only contraceptives (N=4). There was no significant difference in the plasma homocysteine levels at all time-points for the female participants who used either progesterone only contraceptives and combined contraceptives. This finding suggests that hormonal contraception did not impact on plasma homocysteine levels in the sample.

The utility of both plasma homocysteine and serum CDT to act as biomarkers of cardiovascular disease and alcohol consumption respectively, have not been investigated in terms of a identifying a potential correlation between these biomarkers. An association was investigated between the levels of CDT (%) and plasma homocysteine (µmol/l) in the HI study sample. All Spearman's tests were not significant for an association between elevated CDT and plasma homocysteine in the HI study sample.

3.7.2 Plasma Homocysteine Levels within Alcohol Dependent Individuals (ADI)

Plasma homocysteine concentrations at the pre-defined study time-points are represented in figure 3.30.

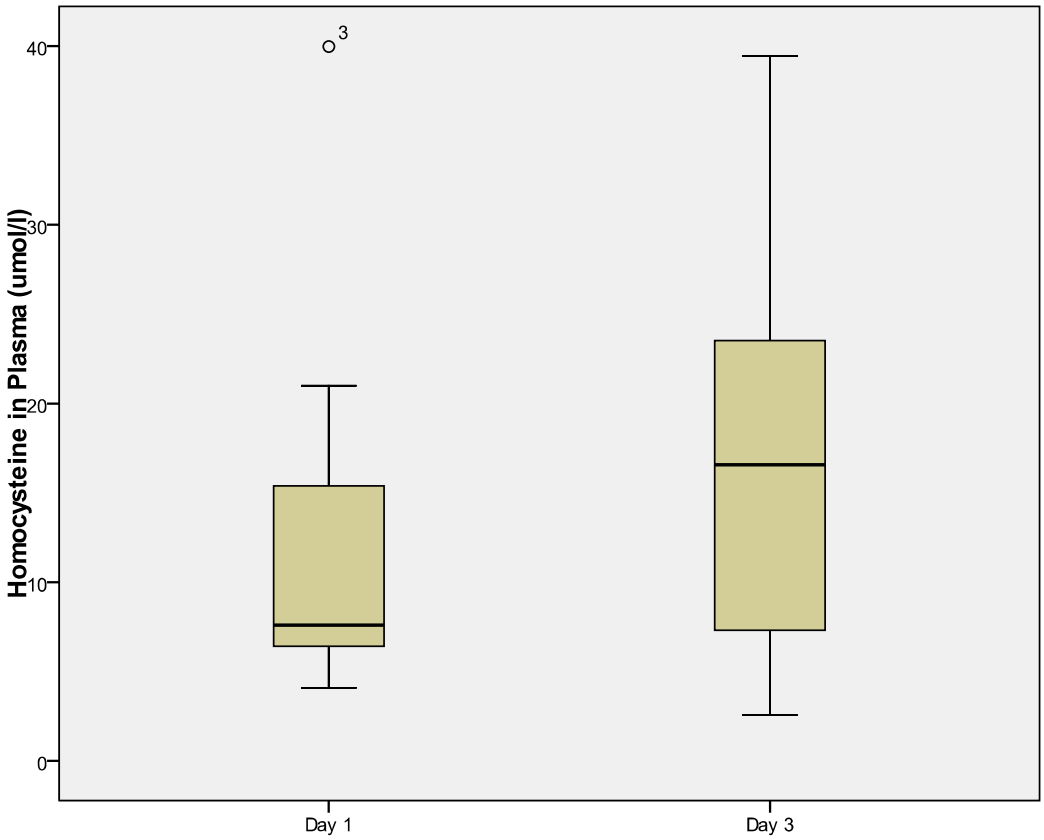

Figure 3.30: Box plots of homocysteine in plasma (µmol/l) for ADI study sample (N=18) *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

No significant difference between homocysteine measured on day 1 and day 3 (p=0.117) was seen. Homocysteine measurement on day 1 or day 3 did not significantly correlate with daily alcohol consumption in grams. No association between homocysteine measured on day 1 and patient age was evident. There was no association between plasma homocysteine and serum CDT, which reflect the result found in the HI study sample.

The effect of smoking on plasma homocysteine levels was also investigated in the ADI study sample. There was a higher number of smokers in the ADI study (N=10), compared to the HI study, however there was no significant difference in the plasma homocysteine levels at each study time-point between smokers and non-smokers, further indicating that smoking did not affect plasma homocysteine. The effect of hormonal contraception could not be investigated in the female participants (N=3) of the ADI study, as no female participants self-reported that they used hormonal contraception.

A possible relationship between homocysteine concentrations and type of alcohol consumed on both days 1 and 3 was investigated and is shown in figure 3.31. Twelve participants consumed one type of alcoholic beverage only. A higher number of participants consumed spirits only (N=6), compared with participants who consumed only cider (N=2) and beer (N=2). From the bar chart shown in figure 3.31, only consumption of beer suggested there was a reduction in plasma homocysteine levels from day 1 to day 3; however this did not take into consideration the amount of alcohol consumed.

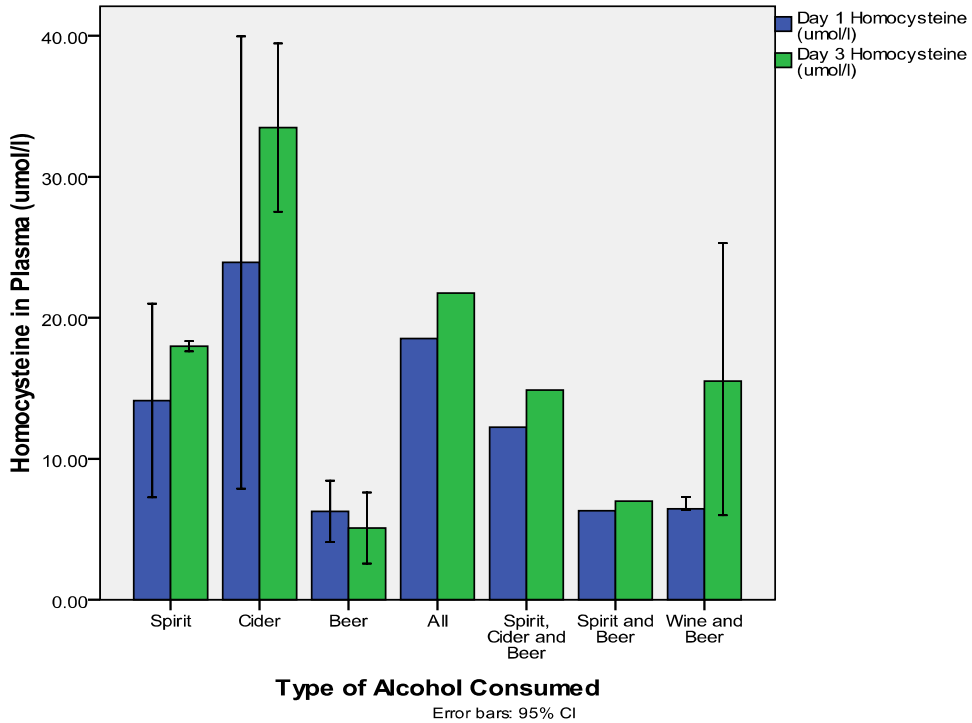

Figure 3.31: Bar chart of plasma homocysteine (µmol/l) in relation to type of alcohol consumed.

3.7.3. Plasma Homocysteine Levels in a Sample of Sessional Drinkers

The range of plasma homocysteine measured at baseline in a sample of sessional drinkers, showed the presence of one outlier and three extreme outliers within the sample.

Figure 3.32 presents the plasma homocysteine levels detected within the group of sessional drinkers in relation to the clinical reference range. The four sessional drinker who were outside of the clinical reference range of 5-15 µmol/l (Antoniades et al. 2009), were evenly split relating to gender, male (N=2), female (N=2), as highlighted in figure 3.32. The four participants who exhibited plasma homocysteine levels above the maximum reference range limit of 15 µmol/l, the plasma homocysteine levels did not significantly change ($p=0.652$) during the study period. For these four outliers their mean alcohol consumption during drinking days did not statistically ($p=0.642$) change for the study period. Furthermore the vitamin co-factors, folate and vitamin B_{12} were also exhibiting low levels, below 5 µg/l for folate and 200-300 ng/l for vitamin B_{12}. For serum folate there was a significant increase ($p<0.05$) during the study duration, however there was no significant increase ($p=0.184$) in vitamin B_{12} levels. This would be consistent with the literature evidence which clearly identifies the link between elevated plasma homocysteine and low levels of vitamin B_{12} and folate (Antoniades et al. 2009). There was a lack of an inverse significant correlation between plasma homocysteine and the serum vitamin co-factors in these outlying participants, however this could be due to the low sample number (N=4) Further analysis of these individuals who were undertaking sessional drinking, highlighted that none of these participates were shown to carry the mutant $MTHFR_{(C677T)}$ polymorphism, further highlighting a potential link between sessional alcohol consumption and elevated plasma homocysteine levels. There was no clinical data, e.g. liver function, blood pressure, cholesterol or lipid levels available, on the aforementioned outliers. It is important to state that as part of the HI study protocol, clinical data on liver function, blood pressure, cholesterol or lipid levels for any participant was not collected or part of the study design.

Although plasma homocysteine is not routinely used as a diagnostic biomarker within UK hospital laboratories, the use of homocysteine as a predictor of

cardiovascular disease risk has been well established in the literature, through numerous laboratory studies and clinical trials (Antoniades et al. 2009; Bonaa et al. 2006; Lonn 2008; Refsum et al. 1998). Many peer reviewed studies have been published showing a clear link between elevated plasma homocysteine and clinical signs of the on-set of cardiovascular disease. Furthermore there is no evidence in the literature that diuresis has an effect on circulating homocysteine levels (Verhoef et al. 2002). This is an important point to consider, as alcohol consumption does have an impact on diuresis.

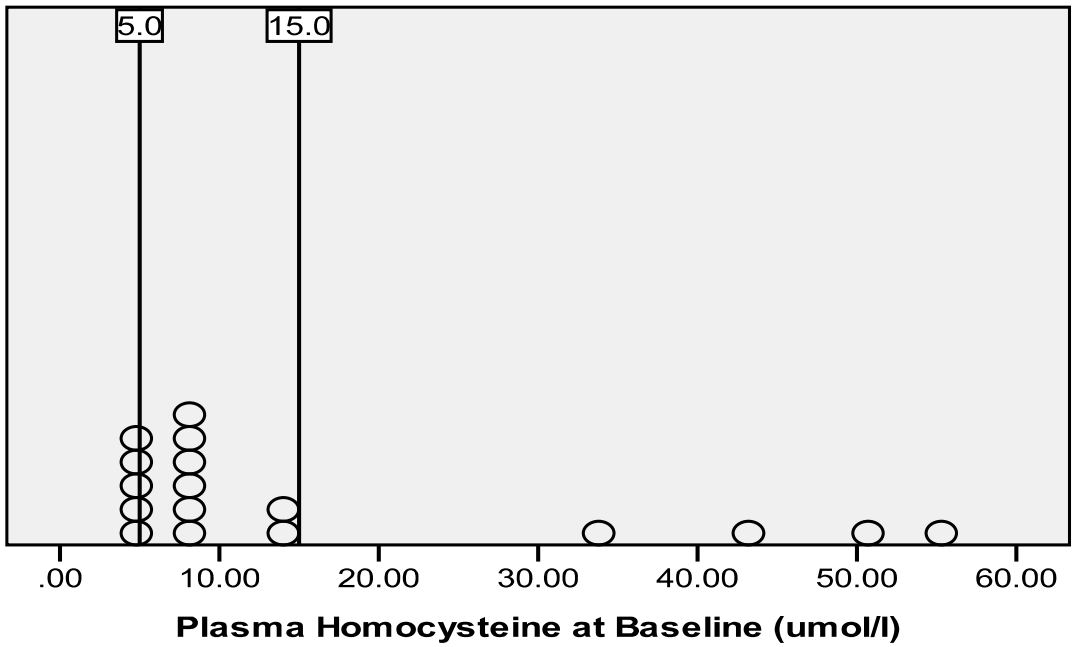

Figure 3.32: Dot plot of sessional drinkers (N=17) plasma homocysteine (µmol/l) levels, in relation to clinical reference range. The vertical reference lines represent the minimum (5 µmol/l) and maximum (15 µmol/l) clinical reference range for plasma homocysteine.

There was no association between plasma homocysteine and mean alcohol consumption during drinking days at baseline in a sample of sessional drinkers, ($p=0.743$, $R=-0.086$). The non-significant correlation was also found at the at 3-month and 6-month time-points.

3.7.4 Plasma Homocysteine Levels within Drinkers Combined Sample (HI and ADI)

The plasma homocysteine, median and range recorded for all drinkers (the HI and ADI study combined samples, not including the abstainers), (N=44) was investigated. There were six outliers within the sample. Figure 3.33 represents each drinker's plasma homocysteine concentration and where it falls within the clinical reference range; 23.53% (N=11) of the sample had plasma homocysteine above 15 µmol/l. Of this percentage 9.1% were ADI study participants and 15.9% were HI study participants.

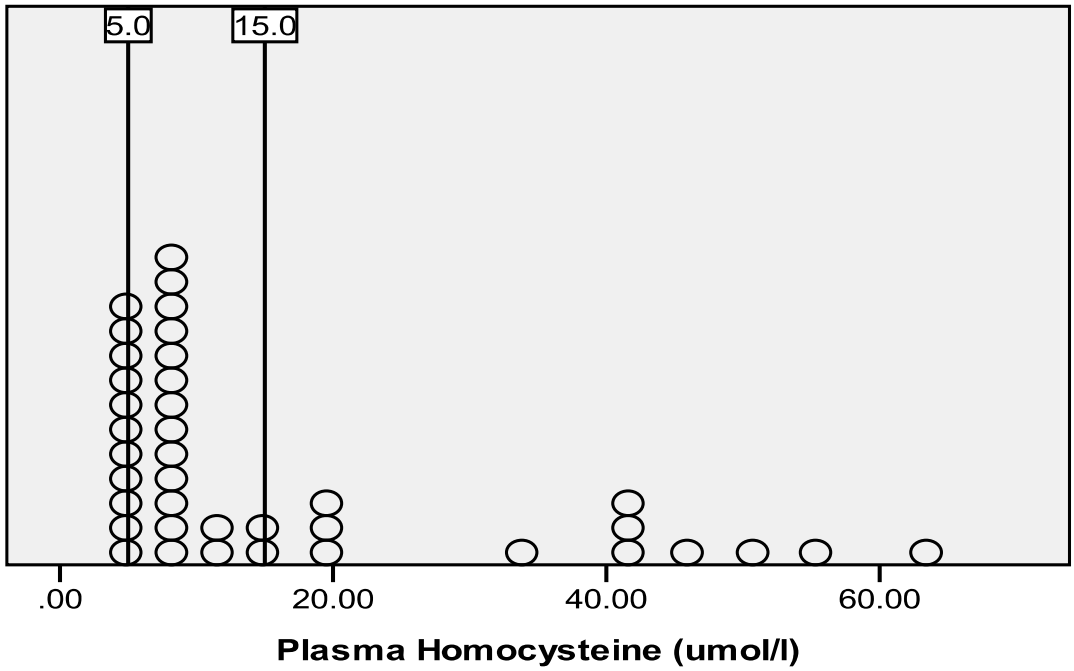

Figure 3.33: Dot plot of homocysteine in plasma (µmol/l) for all drinkers (N=44) in the sample and relationship to clinical reference range. The vertical reference lines represent the minimum (5 µmol/l) and maximum (15 µmol/l) clinical reference range for plasma homocysteine.

There was no significant correlation between daily alcohol consumption in grams and plasma homocysteine levels in a sample of individuals who consume alcohol, regardless of pattern (p=0.795, R=-0.043).

3.7.5 Comparison of Plasma Homocysteine Levels between Sessional Drinkers and Alcohol Dependent Individuals

The median plasma homocysteine levels in sessional drinkers and alcohol dependent patient group were similar, 7.20 µmol/l and 7.89 µmol/l respectively and is illustrated in figure 3.34. Within the box plots shown in figure 3.34, the sessional drinkers group did shown 3 extreme outliers and 1 outlier within the sample, compared with just one outlier within the alcohol dependent patient sample. There was no significant difference between the levels of homocysteine in each patient group (p=0.818).

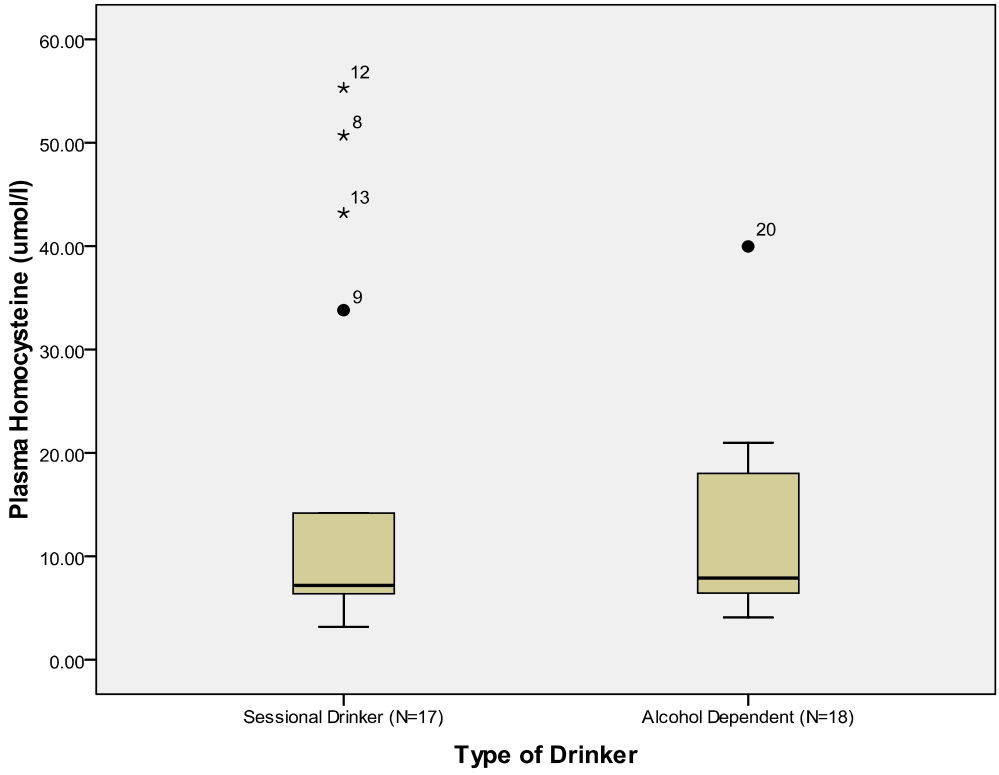

Figure 3.34: Box plots of homocysteine in plasma in a sample of sessional drinkers compared to alcohol dependent patients *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

3.8 Homocysteine in Urine: Method Development

As described in chapter 2, method development for the detection of homocysteine in human urine was undertaken. In published literature homocysteine is analysed within serum and plasma routinely, however, methods have also been published for the detection of homocysteine in urine (Thomson and Tucker 1986; Thomson and Tucker 1985). Urine is a less invasive biological fluid to collect, reducing the need to carry out venepuncture. A detection method for homocysteine in urine would be useful clinically and for research.

In the published method (Thomson and Tucker 1986) for the detection of homocysteine in urine, the result was expressed as homocysteine per milligrams of creatinine. Therefore every urine sample analysed for homocysteine was also analysed for creatinine concentration using the Jaffe method as described in chapter 2. Creatinine is commonly used to normalise urinary markers however there are problems associated with the sensitivity and efficiency of the assay (Waikar et al. 2010). The problems associated with creatinine levels, can be induced through injury to the kidney which disrupts urine production, therefore causing an inbalance in creatinine kinetics and urine excretion, therefore resulting in the underestimation of both creatinine and the subsequent biomarker (Waikar et al. 2010). It is important to note that no participants in either study (HI and ADI) self-reported kidney injury or problems with urination. There are other substances used to determine normal urinary excretion, which includes p-aminobenzoic acid (PABA). The use of PABA can determine if urine 24 hour collection has been completed by determining the recovery of the compound. However this involves measuring PABA which was ingested and both the HI and ADI studies did not ask participants to ingest PABA to determine its recovery and as only a single waking urine sample was required, the use of PABA was not justified. The use of creatinine to normalise urinary homocysteine and other alcohol biomarkers has been well established in the literature (Bergström et al. 2003; Kusmierek et al. 2006; Thomson and Tucker 1986).

3.8.1 Calibration

A calibration standard curve of homocysteine was carried out using the same procedure as described for the analysis of homocysteine in plasma. The calibration graph of an aqueous solution of homocysteine was linear in the concentration range of 0-20 µmol/l. The equation of the linear graph was y=25241X+114619 and the coefficient of determination for the calibration was R^2=0.99. The calibration graph is shown in figure 3.35.

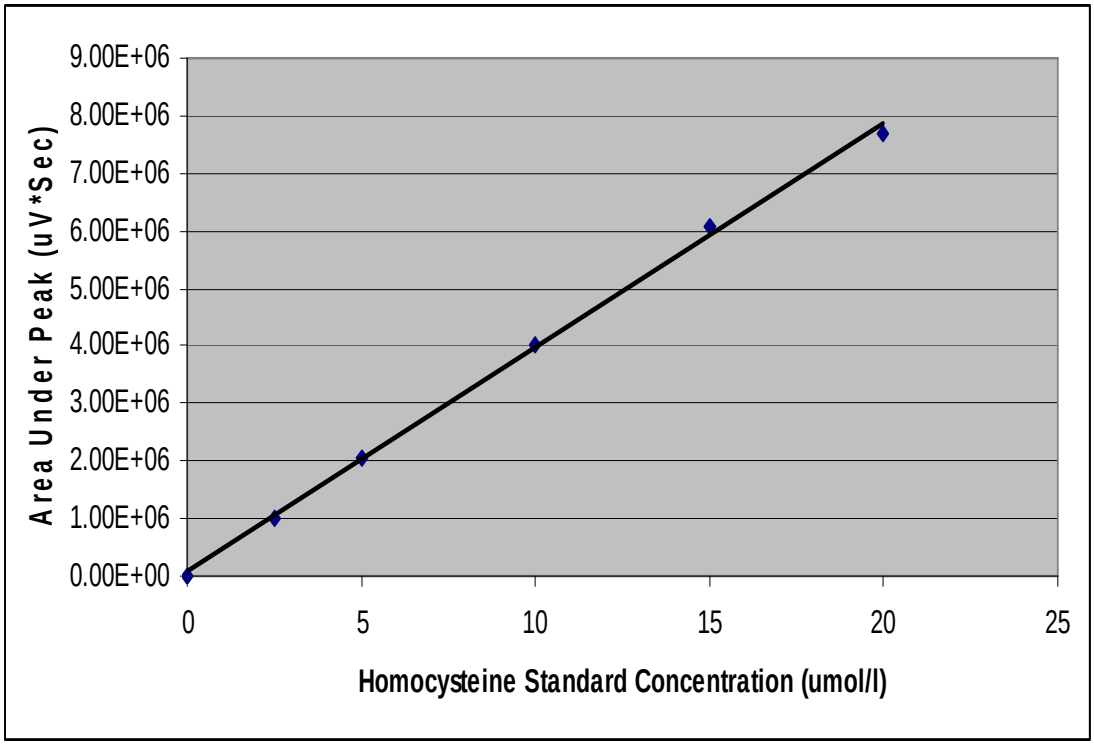

Figure 3.35: Calibration of homocysteine measured in urine

3.8.2 Results

All samples were analysed for homocysteine in urine, both from the HI and ADI studies. HPLC runs were conducted in batches and in duplicate as described in the method chapter. Homocysteine expressed in urine, eluted form the HPLC column at approximately 8 minutes, which is consistent with the retention time stated in the peer-reviewed method paper (Thomson and Tucker 1986). The retention time for urine was different from plasma homocysteine analysis, by a difference of two minutes, meaning the homocysteine peak detected in urine elutes from the column

earlier. The potential reasons for this could be based in the different types of biological fluid and their interaction with the column binding sites, causing a faster elution profile. Figures 3.36 and 3.37 are examples of chromatograms with a positive homocysteine detection and negative homocysteine detection in urine respectively.

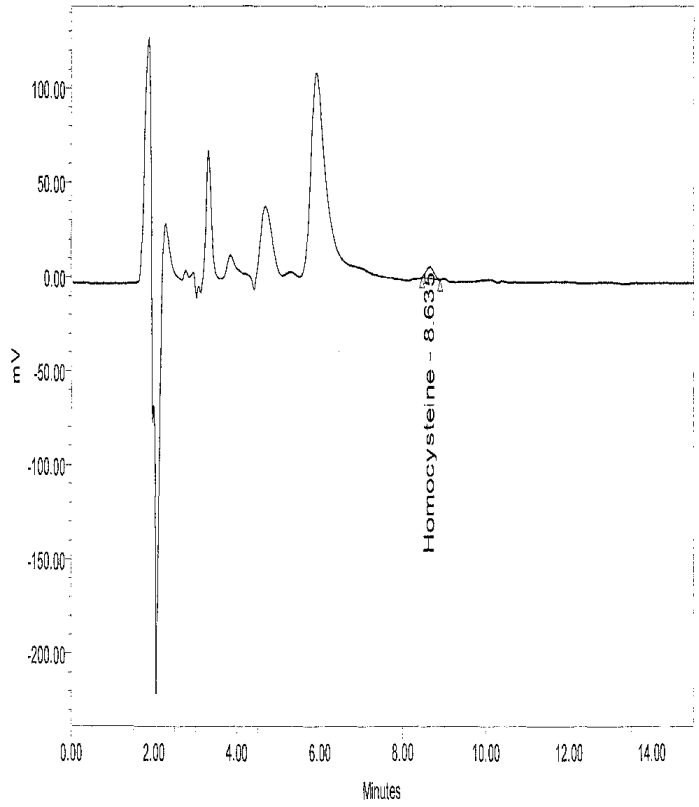

Figure 3.36: Positive Homocysteine in Urine Chromatogram

Detector: electrochemical; volume injected: 20 µl; flow rate: 0.8 ml/min; sensitivity setting: +0.86 volts; total run time: 15 minutes.

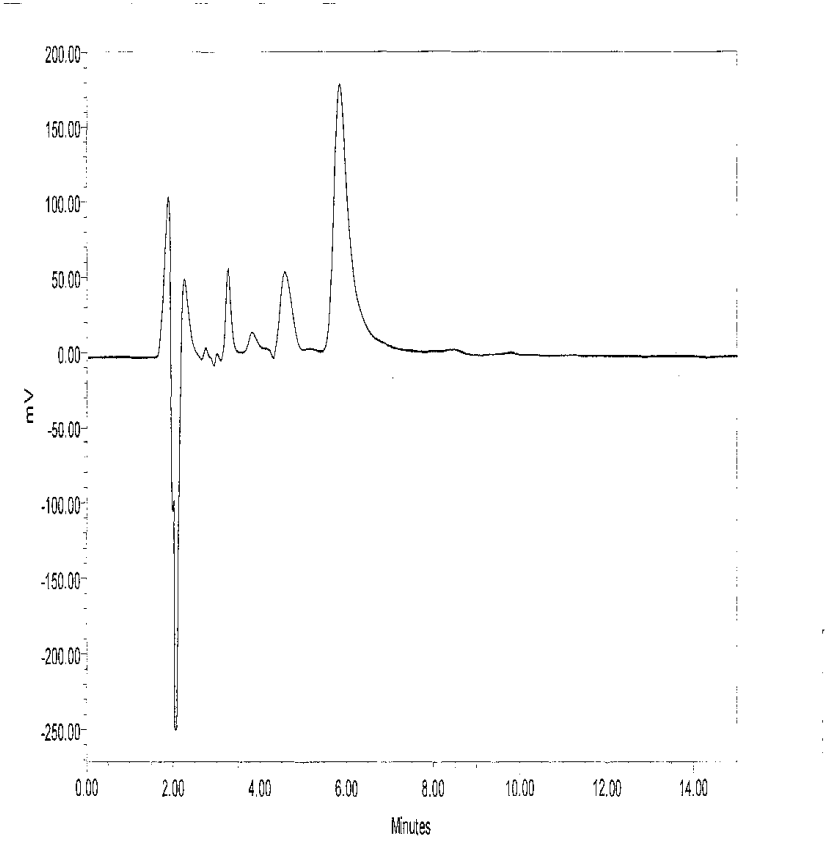

Figure 3.37: Negative Homocysteine in Urine Chromatogram

Detector: electrochemical; volume injected: 20 µl; flow rate: 0.8 ml/min; sensitivity setting: +0.86 volts; total run time 15 minutes.

There were a total of 35 HI participant urine samples analysed in duplicate and from these samples, 22.9% (N=8) generated a peak for homocysteine at the anticipated retention time. Over 75% of the urine samples did not contain detectable levels of homocysteine. Table 3.10 details the HI study samples and homocysteine levels in urine with additional urinary creatinine concentrations.

Table 3.10: HI study levels of homocysteine in urine

Participant Number	Time point	Urinary Homocysteine (µmol/l)	Urinary Creatinine (mmol/l)	Homocysteine/Creatinine Ratio
11	Baseline	1.82	1.86	0.98
25	3-month	0.82	17.15	0.05
30	3-month	1.51	12.29	0.12
31	3-month	0.54	9.19	0.06
31	6-month	1.33	4.33	0.31
32	Baseline	0.92	18.48	0.05
33	3-month	0.89	7.87	0.11
35	3-month	1.76	8.04	0.22
35	6-month	1.48	6.81	0.22
36	3-month	0.52	7.16	0.07
36	6-month	0.65	6.45	0.10

Within the ADI study, six participant samples contained detectable levels of homocysteine in urine. This represents 38.9% of the total sample analysed (N=18). Table 3.11 shows the detectable levels of homocysteine in urine with urinary creatinine concentrations.

Table 3.11: ADI Study levels of homocysteine in urine

Participant Number	Time point	Urinary Homocysteine (µmol/l)	Urinary Creatinine (mmol/l)	Homocysteine/Creatinine Ratio
102	Day 1	0.24	21.22	0.10
111	Day 1	1.25	19.80	0.56
111	Day 3	1.26	7.78	1.43
112	Day 3	0.35	25.55	0.12
114	Day 1	0.73	15.03	0.43
114	Day 3	0.50	21.48	0.21
115	Day 3	1.19	7.96	1.32
117	Day 3	0.39	16.80	0.21

Further work was undertaken to investigate if the ratio of urinary homocysteine to creatinine was associated with daily alcohol consumption, expressed in grams and the alcohol biomarker CDT, measured in serum. CDT results were not available for all study participants. There was no association between %CDT in serum and urinary homocysteine/creatinine ratio, for the study sample, however the sample size was very small (N=5), which is not a large enough sample size for correlation analysis.

There was a significant inverse correlation between the daily alcohol consumption in grams and urinary homocysteine/creatinine ratio in the sample of ADI and HI study participants found to express homocysteine in urine (N=19) (figure 3.38).

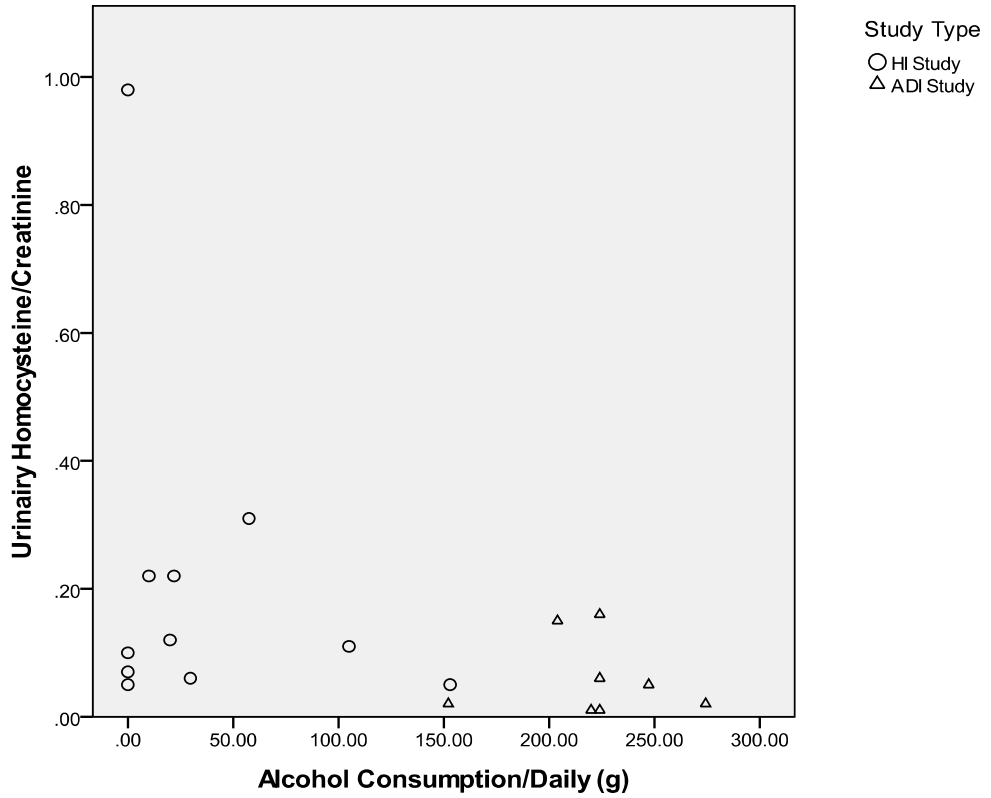

Figure 3.38: Scatter plot of association between daily alcohol consumption and urinary homocysteine/creatinine ratio in the sample of HI (N=11) and ADI (N=8) study participants with homocysteine expressed in their urine (p=0.038, R=-0.480 Spearman's test)

Further investigation was carried out to determine if there was an association between plasma homocysteine and the ratio of homocysteine/creatinine levels in urine, shown in figure 3.39. No significant correlation (p=0.107, R=-0.381) between the levels of plasma homocysteine and the levels of homocysteine expressed as a ratio with creatinine in urine were evident.

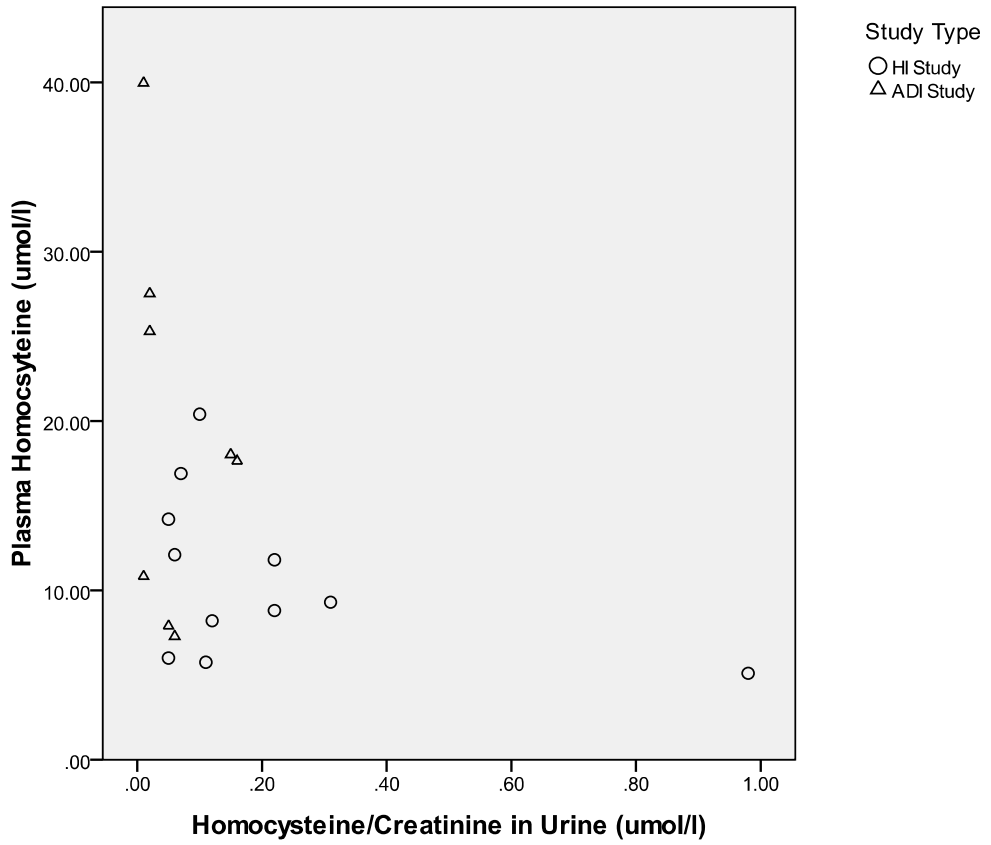

Figure 3.39: Scatter plot of homocysteine/creatinine ratio plotted as a function of plasma homocysteine (p=0.107, R=-0.381 Spearman's test)

3.8.3 Summary

The results from this method development indicates that the HPLC method outlined does show potential (detection in nineteen samples) however more work within this area is required to determine if this method can produce an accurate and more sensitive determination of homocysteine in urine. An association was investigated to determine if there was a link between the levels of plasma homocysteine and urinary homocysteine expressed as a ratio with creatinine. No association was evident; however this could improve with a more sensitive urinary homocysteine assay. Further investigation is required to develop a sensitive HPLC assay for the detection of homocysteine in urine, which can be utilised as a biomarker of alcohol consumption.

3.9 Plasma Homocysteine and the MTHFR$_{(C677T)}$ Polymorphism

Since the presence of the mutant form on the MTHFR$_{(C677T)}$ polymorphism can promote an increase in circulating plasma homocysteine, it is essential to determine its existence when investigating the cause of observed changes in plasma homocysteine levels.

3.9.1 Prevalence of the MTHFR$_{(C677T)}$ Polymorphism within Healthy Individuals (HI) who Consume Alcohol in a Range of Drinking Patterns

The prevalence of the MTHFR$_{(C677T)}$ polymorphism in relation to participant's alcohol drinking pattern is shown in table 3.12; 17.1% (N=6) of the study population tested positive for the mutant MTHFR$_{(C677T)}$ polymorphism.

Table 3.12: Prevalence of the MTHFR$_{(C677T)}$ polymorphism within the HI study participants (N=35), categorised according to baseline alcohol drinking pattern

Genotype	Abstainer (N=7)	Responsible Drinker (N=2)	Exceeding Responsible Guidelines but NOT Sessional Drinker (N=8)	Sessional Drinker (N=18)
Heterozygote (N=11)	3	0	0	8
Wild-Type Homozygote (N=18)	2	1	6	9
Mutant Homozygote (N=6)	2	1	2	1
Total (N=35)	7	2	8	18

Six HI study participants were found to have the mutant MTHFR$_{(C677T)}$ polymorphism, however only five participants consented for their biological samples to be analysed to investigate specifically the impact of the mutant polymorphism on levels of plasma homocysteine. This explains the reduction in sample number from

six to five participants. The concentration of plasma homocysteine found in the study sample in relation to the presence of the MTHFR$_{(C677T)}$ polymorphism and alcohol drinking pattern is shown in figure 3.40. Plasma homocysteine levels did not significantly increase (p=0.165) at each pre-defined study time-point in the individuals who were found to have the mutant MTHFR$_{(C677T)}$ polymorphism.

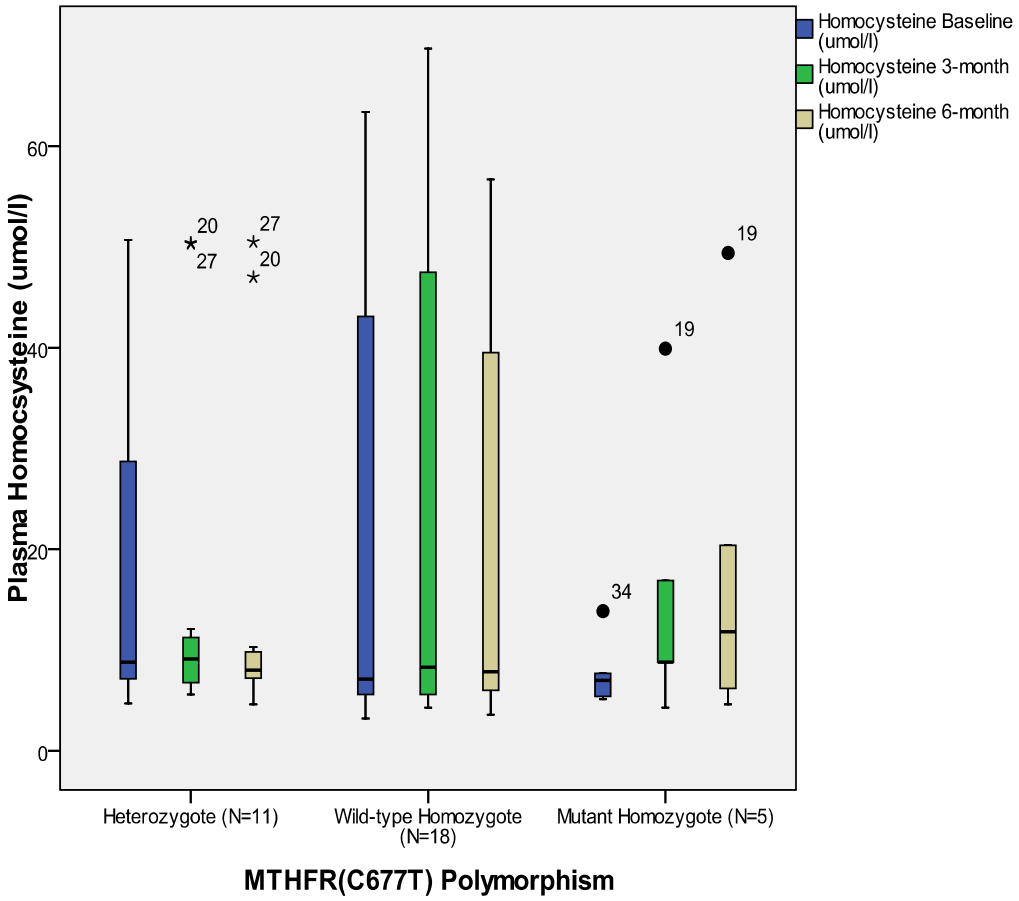

Figure 3.40: Box plots of plasma homocysteine (µmol/l) at different time points for individual genotyped according to MTHFR$_{(C677T)}$ polymorphism *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

In relation to serum folate levels, which like plasma homocysteine levels, are linked to the presence of the mutant MTHFR$_{(C677T)}$ polymorphism, only one participant with the mutant MTHFR$_{(C677T)}$ polymorphism was found to have serum folate levels below the minimum clinical reference range of 5 µg/l at the baseline and 3-month time-

point. However serum folate increased to greater than 5 µg/l by the 6-month time-point. All other participants (N=4), had serum folate levels within the clinical reference range at all time-points. In relation to vitamin B_{12} levels, all participants who were found to carry the mutant $MTHFR_{(C677T)}$ polymorphism had vitamin B_{12} levels within the clinical reference range of 200-900 ng/l.

The association between plasma homocysteine concentrations in relation to the grams of alcohol consumed per drinking day at baseline, by each of the study participants and the subsequent genotyping for the $MTHFR_{(C677T)}$ polymorphism was investigated (table 3.13). The ratio of plasma homocysteine to mean alcohol consumption during drinking days was significantly higher ($p<0.05$) in the participants who were genotyped for the mutant $MTHFR_{(C677T)}$ polymorphism.

Table 3.13: Drinking characteristics of different $MTHFR_{(C677T)}$ polymorphism genotypes found in the HI study (*denotes significance)

Baseline Time-Point	**Heterozygote (N=11)**	**Wild-Type Heterozygote (N=18)**	**Mutant Homozygote (N=5)**
Plasma Homocysteine (µmol/l) Median, Mean (SD; Range)	8.8, 18.5 (18.0; 4.7-50.7)	6.7, 17.6 (20.4; 3.2-63.4)	7.7, 8.4 (3.3; 5.1-13.8)
Mean alcohol consumption per drinking day (g) Median, Mean (SD; Range)	48.0, 55.9 (52.4; 0.0-153.0)	30.2, 33.5 (26.6; 0.0-92.5)	20.0, 33.4 (48.5; 0.0-117.0)
Ratio of median plasma homocysteine to median alcohol consumption (g) per drinking day	0.18	0.22	0.35*

No significant association was found between plasma homocysteine at baseline and study participants baseline mean alcohol consumption during drinking days in participant's who were genotyped as heterozygote ($p=0.552$) and wild-type homozygote ($p=0.538$) for the $MTHFR_{(C677T)}$ polymorphism. However a significant correlation was revealed between plasma homocysteine at baseline and mean

alcohol consumption during drinking days for participant's genotyped as mutant homozygote for the $MTHFR_{(C677T)}$ polymorphism. From figure 3.41 a significant linear relationship is identified in participants who carry the mutant $MTHFR_{(C677T)}$ polymorphism ($p<0.05$, $R=0.975$). In this group increasing alcohol consumption was associated with increasing plasma homocysteine levels.

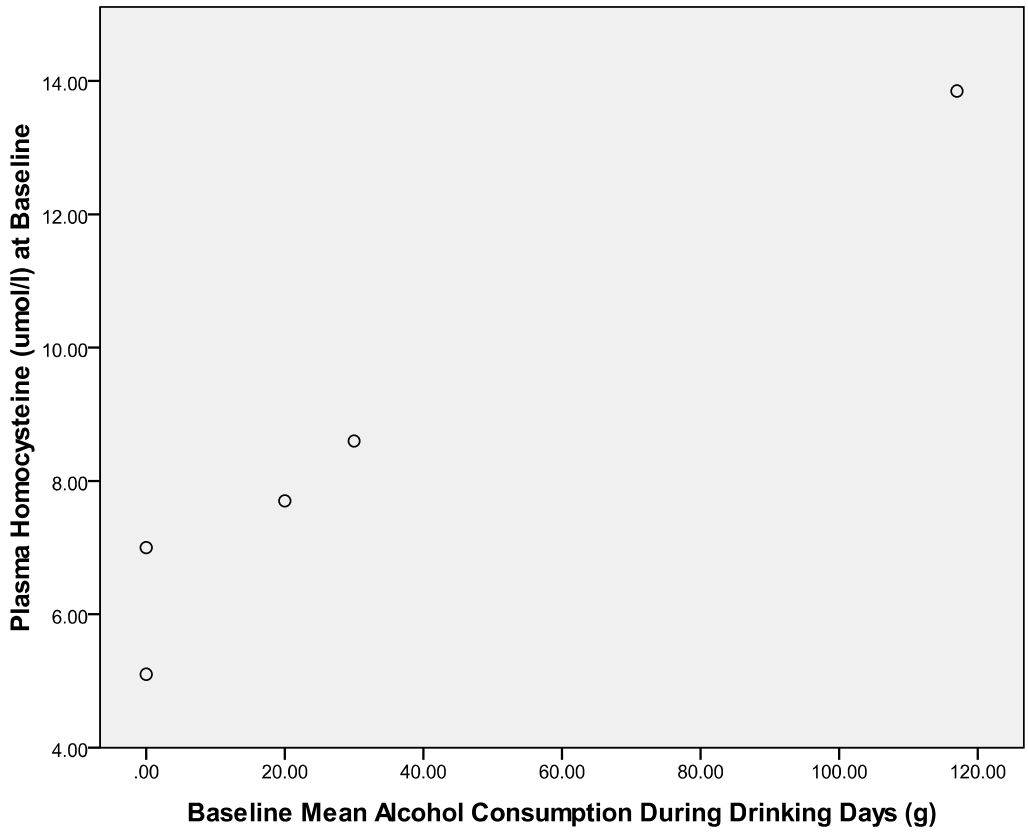

Figure 3.41: Homocysteine in plasma (µmol/l) in relation to baseline alcohol consumption (g) in a sample of HI study participants (N=5) with the mutant $MTHFR_{(C677T)}$ polymorphism ($p<0.05$, $R=0.975$ Spearman's test)

3.9.2 Prevalence of the MTHFR$_{(C677T)}$ Polymorphism within Alcohol Dependent Individuals (ADI)

The patients who participated in this study were genotyped for the MTHFR$_{(C677T)}$ polymorphism, the results of which are shown in table 3.14. Two participants did not provide sufficient blood samples for DNA extraction.

Table 3.14: Frequency of MTHFR$_{(C677T)}$ Polymorphism within study sample

	Heterozygote	Wild-Type Homozygote	Mutant Homozygote
Number of Participants	6	10	0

No patients within the ADI study were found to carry the mutant MTHFR$_{(C677T)}$ polymorphism. The homocysteine concentrations on day 1 and day 3, in relation to the MTHFR$_{(C677T)}$ polymorphism are shown in figure 3.42.

There was no significant difference in plasma homocysteine levels between days 1 and 3 in ADI participants genotyped as having either the heterozygote or wild type homozygote MTHFR$_{(C677T)}$ polymorphism. There was also no significant difference in plasma homocysteine levels on days 1 and 3 between the two different MTHFR$_{(C677T)}$ genotypes. The result suggests that the wild-type homozygote and heterozygote variants of the MTHFR gene did not affect the plasma homocysteine levels in a sample of dependent individuals.

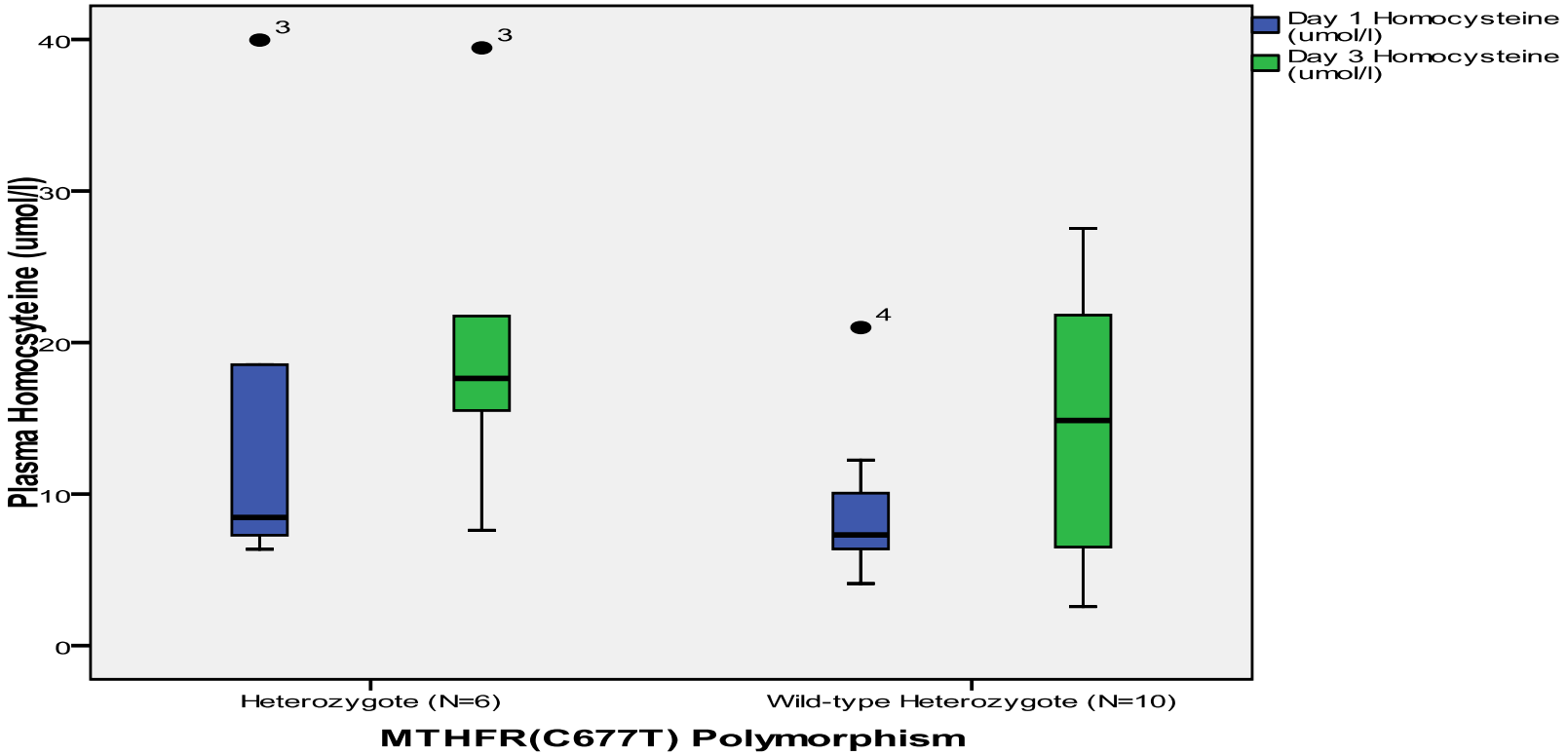

Figure 3.42: Box plots of homocysteine in plasma (µmol/l) for ADI study (N=16) sample in relation to prevalence of $MTHFR_{(C677T)}$ polymorphism at each study time-point *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

3.9.3 Prevalence of the MTHFR$_{(C677T)}$ Polymorphism: Comparison of Sessional Drinkers and Alcohol Dependent Individuals

The prevalence of the MTHFR$_{(C677T)}$ polymorphism was identified in both the sample of sessional drinkers and alcohol dependent patients and is illustrated in table 3.15.

Table 3.15: Frequency of MTHFR polymorphism in sub-analysis sample

	Sessional Drinkers (N=17)	Alcohol Dependent Patients (N=16)*
Heterozygote	8	6
Wild-type	8	10
Mutant	1	0

*Two ADI study participants did not provide sufficient blood sample for DNA extraction.

The plasma homocysteine levels in relation to the MTHFR$_{(C677T)}$ genotyping are illustrated in figure 3.43. The plasma homocysteine levels for the participant who carried the mutant MTHFR$_{(C677T)}$ polymorphism was 13.85 µmol/l, which was higher in comparison to the other MTHFR$_{(C677T)}$ genotypes, in both the alcohol dependent individuals and sessional drinkers. It was not possible to compare the mutant MTHFR$_{(C677T)}$ polymorphism within the alcohol dependent patient group, due to no participant carrying the mutant gene. There was no significant difference (p=0.380) between the plasma homocysteine levels found in the participants who carried either the heterozygote and wild-type genotypes.

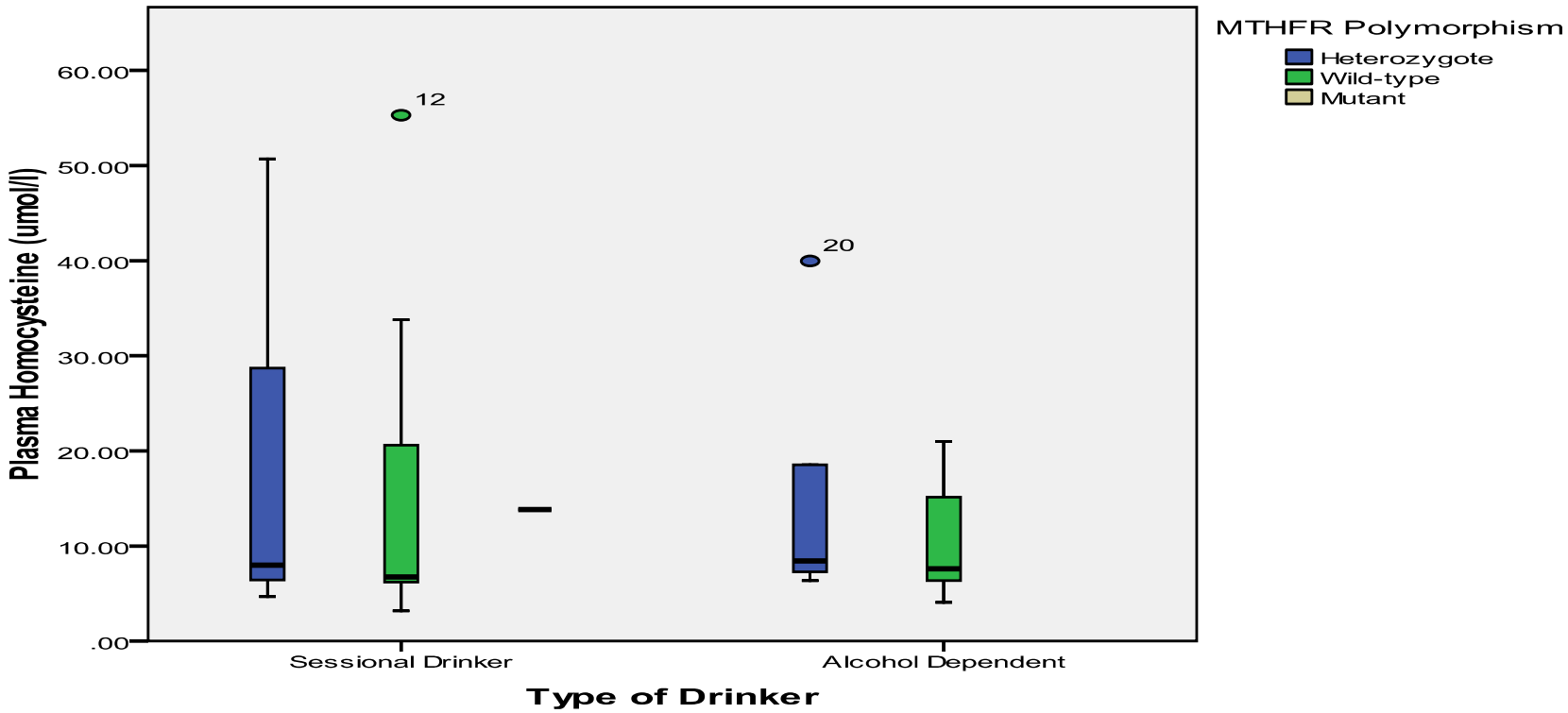

Figure 3.43: Box plots of plasma homocysteine levels (µmol/l) in relation to type of drinker and $MTHFR_{(C677T)}$ genotyping *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box..*

3.10 Homocysteine Vitamin Co-Factors and Alcohol Consumption

The remethylation of homocysteine to methionine is mediated by methionine synthase using two vitamins, folate and vitamin B_{12}. A lack of folate and vitamin B_{12} in this reaction, causes elevated circulating levels of homocysteine in the plasma or serum.

3.10.1 Serum Folate Levels within Healthy Individuals (HI) who Consume Alcohol in a Range of Patterns

Folate, was measured in serum using the AVIDA method at the biochemistry laboratories within the Royal Infirmary of Edinburgh (RIE), as described in chapter 2. The concentrations of serum folate detected in each alcohol drinking pattern is represented in figure 3.44.

Serum folate was shown to increase over the study duration, which could be linked to the decrease in alcohol consumption, which was also found. Serum folate levels significantly increased over the study duration for abstainers and sessional drinkers ($p=0.016$ and $p=0.005$ respectively). This significant increase was not found in the other two drinking pattern groups. Since the remethylation of homocysteine to methionine is dependent on folate as a cofactor, a correlation analysis was performed between baseline serum folate and baseline plasma homocysteine levels but was not found to be significant ($p=0.61$, $R=-0.324$). The participants with elevated plasma homocysteine but low serum folate levels shown in figure 3.45, all had various drinking patterns, four were females and four were males, and none of the participants were found to carry the mutant $MTHFR_{(C677T)}$ polymorphism. However five of the participants did consume alcohol out with the responsible drinking guidelines. There was no significant correlation between plasma homocysteine and serum folate for any of the alcohol drinking patterns at baseline.

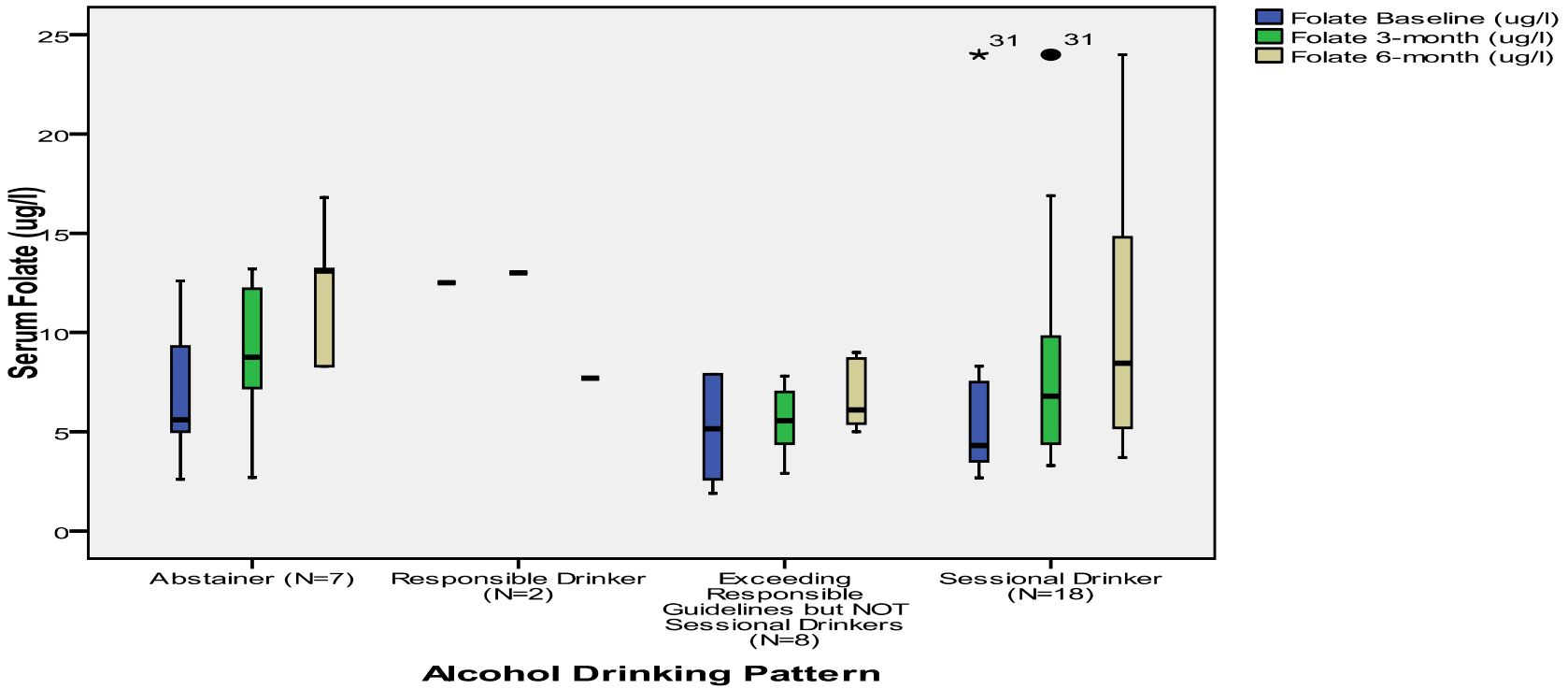

Figure 3.44: Box plots of serum folate (µg/l) for HI study (N=35) sample, categorised according to alcohol drinking pattern over study duration *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

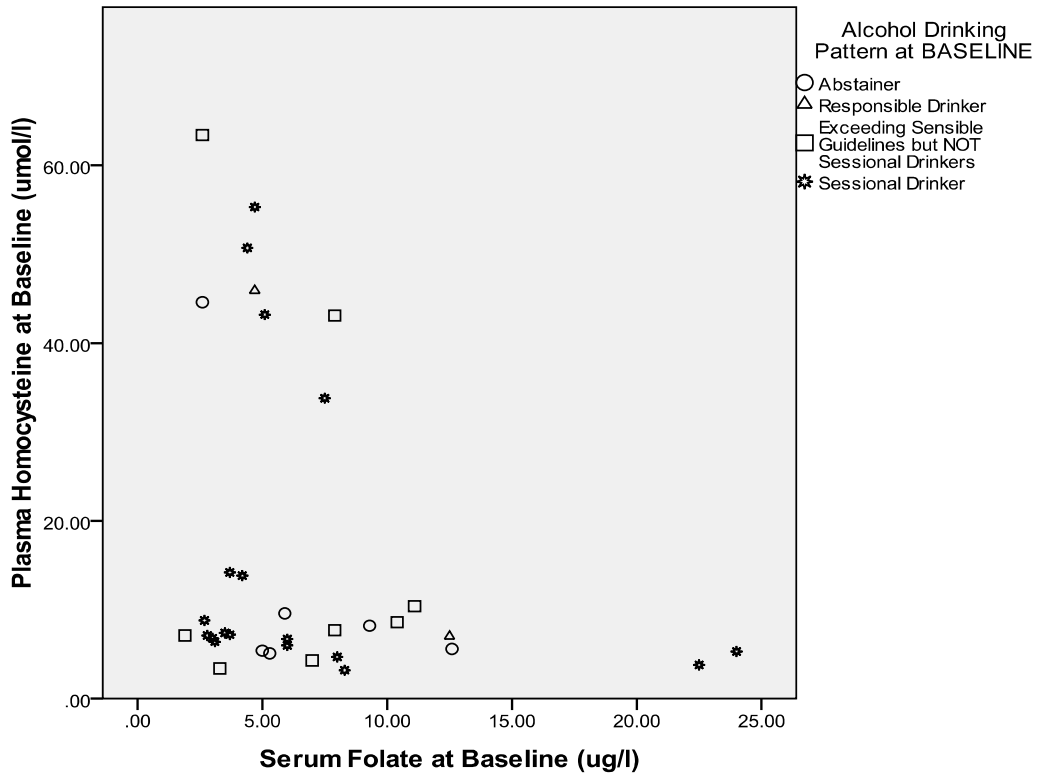

Figure 3.45: Scatter plot of serum folate and plasma homocysteine in a sample of healthy individuals (N=35) with a range of alcohol drinking patterns

The dot plot, shown in figure 3.46 represents the folate concentration in relation to the clinical reference range. Within the sample 40.6% of individuals had serum folate levels below the 5.0 µg/l minimum reference range. Of this group more were female (N=9) compared to male (N=5). Of the study participants (N=14), who had folate levels below 5.0 µg/l, 66.7% were sessional drinkers. Of the remaining participants, 13.3% exceeded responsible guidelines but were not sessional drinkers and 6.6% were abstaining individuals.

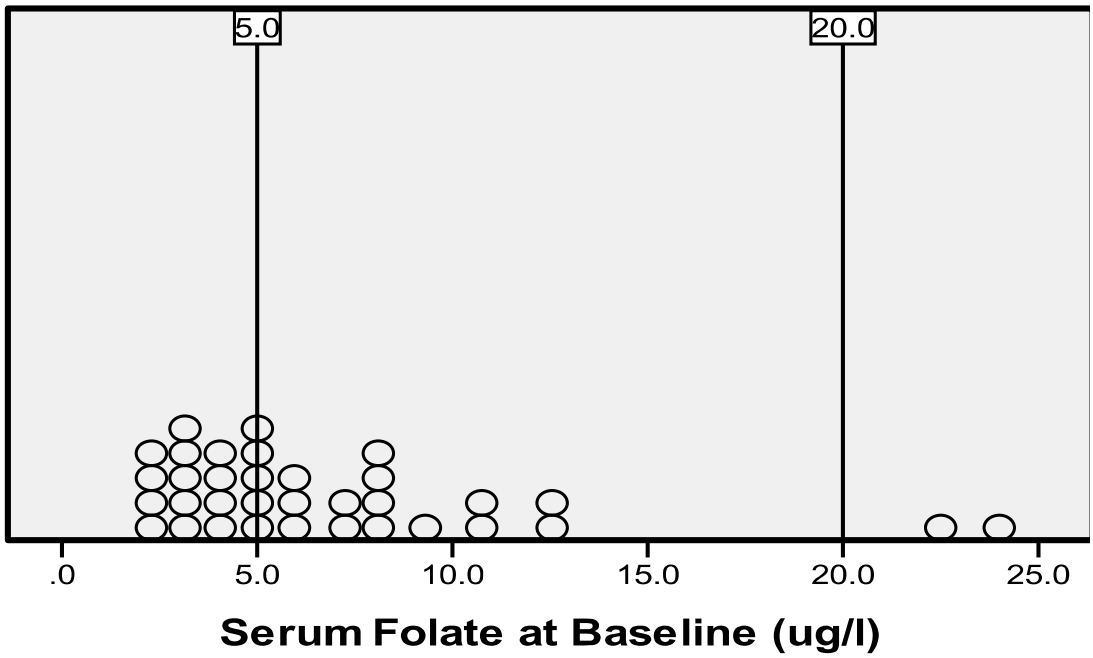

Figure 3.46: Dot plot of serum folate (µg/l) levels within HI Study (N=35) sample, in relation to clinical reference range. The vertical reference lines represent the minimum (5 µg/l) and maximum (20 µg/l) clinical reference range for serum folate.

3.10.2 Serum Folate Levels within Alcohol Dependent Individuals (ADI)

The concentrations of folate identified in samples are shown in figure 3.47.

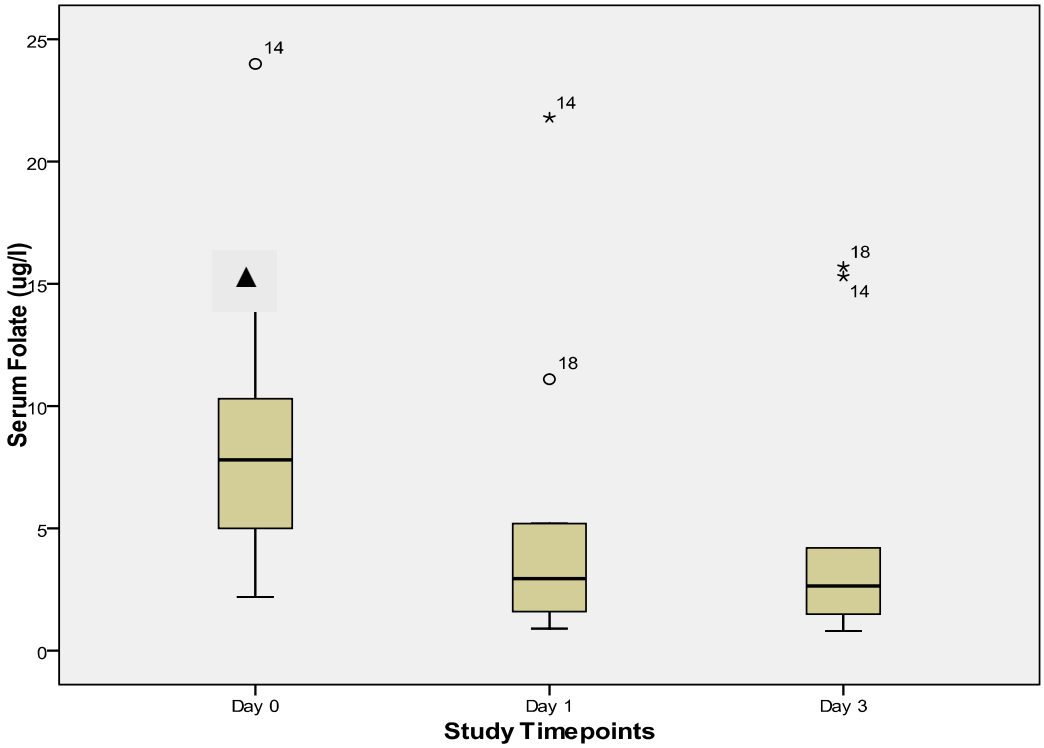

Figure 3.47: Box plots of folate in serum (µmol/l) in ADI study sample at the predetermined study time-points (▲p=0.007, Friedman's test) *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score).* ● *= Outlier (More than 1.5 box lengths above or below the box).* * = *Extreme case (more than 3 box lengths above or below the box.*

A significant reduction in the levels of folate measured on days 0, 1 and 3 (p=0.007) was seen, highlighting a change in folate during the first 72 hours of alcohol detoxification, as shown in figure 3.47. For most participants, serum folate was shown to decrease as detoxification treatment progressed. However this was not true in the group of participants who consumed wine and beer prior to detoxification (figure 3.48), however the increase was not significant (p=1.000). This non significant increase in serum folate levels was not found in the two ADI participants who consumed only beer. Participants were not asked which type of wine they consumed, i.e. red, white or rose).

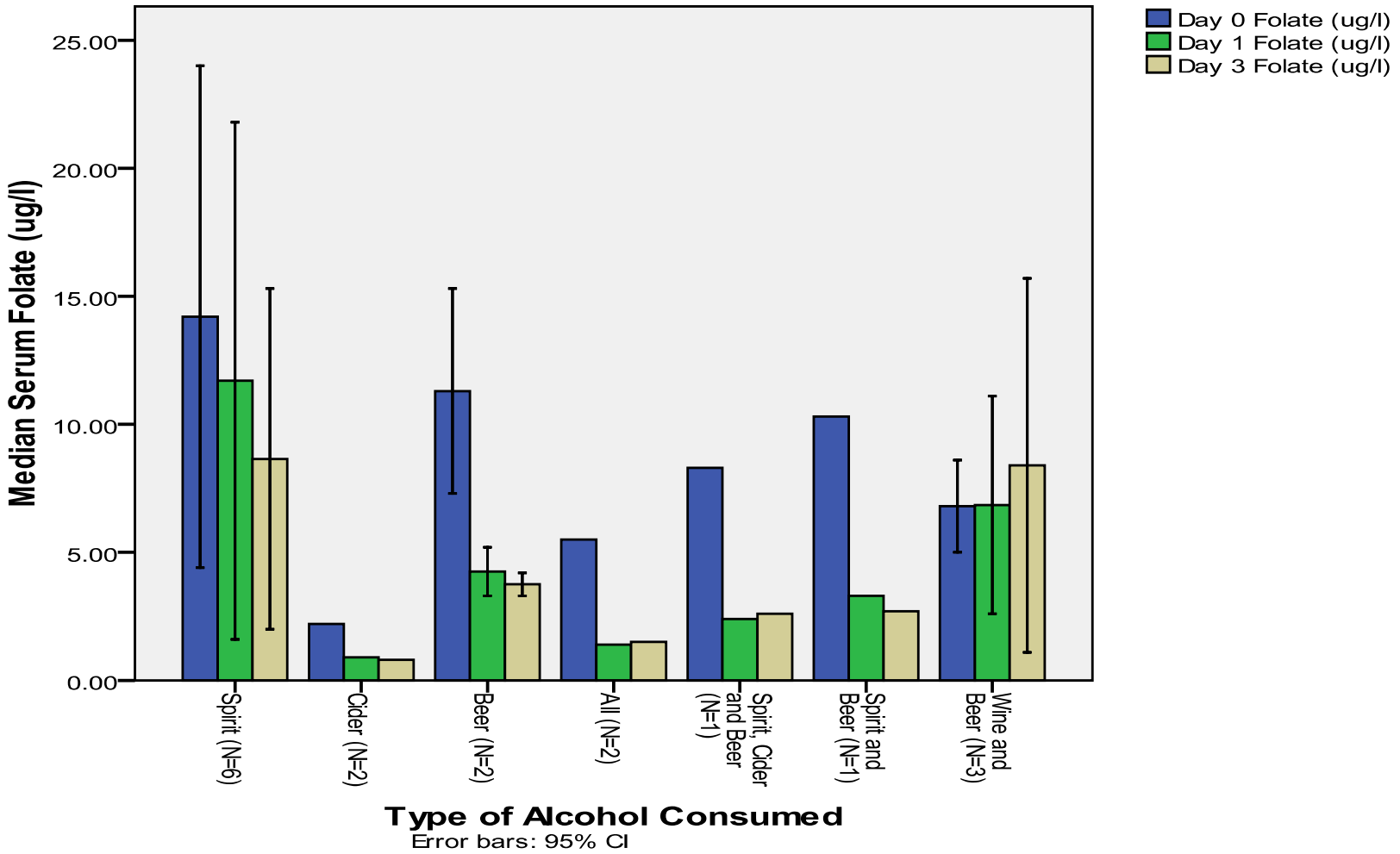

Figure 3.48: Serum folate (µg/l) of ADI study sample during detoxification in relation to type of alcohol consumed.

Serum folate has an inverse relationship with plasma homocysteine concentrations, as folate acts as a methyl donor during the remethylation of homocysteine to methionine (Antoniades et al. 2009). If folate is not present during the remethylation reaction, homocysteine cannot be converted to methionine, causing an increase in circulating homocysteine. There was no correlation found between plasma homocysteine and serum folate measured on day 1 (p=0.140, R=-0.452). The correlation was repeated for serum folate and plasma homocysteine measurements on day 3; which was also not significant, (p=0.154, R=-0.460).

A dot plot (figure 3.49) of each ADI participant's serum folate concentration was created to identify the percentage of the study sample which was within the acceptable folate clinical reference range (5-20 µg/l); 35.29% of the study sample were below the minimum reference folate level of 5 µg/l. The percentage of study participants who had folate levels below the minimum clinical reference range of 5 µg/l increased to 61.5% at day 1 and 81.8% at day 3, which confirms that folate levels were reducing during alcohol detoxification. There was no significant correlation between the serum folate levels and amount of alcohol consumed prior to detoxification on day 3 (p=0.498, R=-0.229).

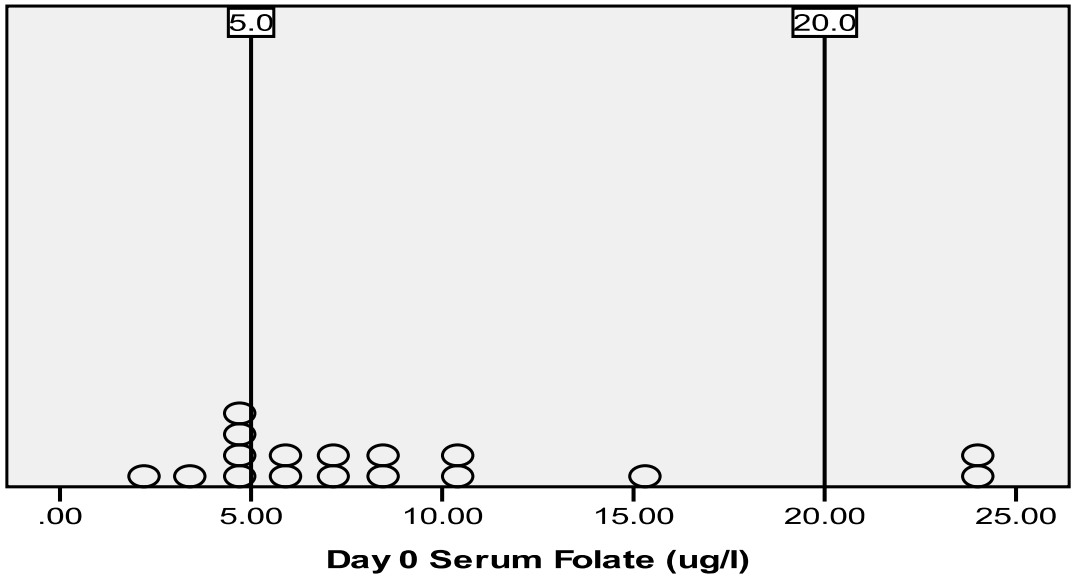

Figure 3.49: Dot plot of serum folate (µg/l) in ADI study (N=18) sample, in relation to clinical reference range. The vertical reference lines represent the minimum (5 µg/l) and maximum (20 µg/l) clinical reference range for serum folate.

3.10.3 Serum Folate Levels within a Comparison of Sessional Drinkers and Alcohol Dependent Individuals

The median serum folate level in sessional drinkers was 4.70 µg/l compared with alcohol dependent individuals group, which was 3.30 µg/l (Figure 3.50). Both median values are below the lower limit of the clinical reference range of 5 µg/l. There was no significant difference between the levels of folate in a sample of sessional drinkers compared to alcohol dependent individuals (p=0.116).

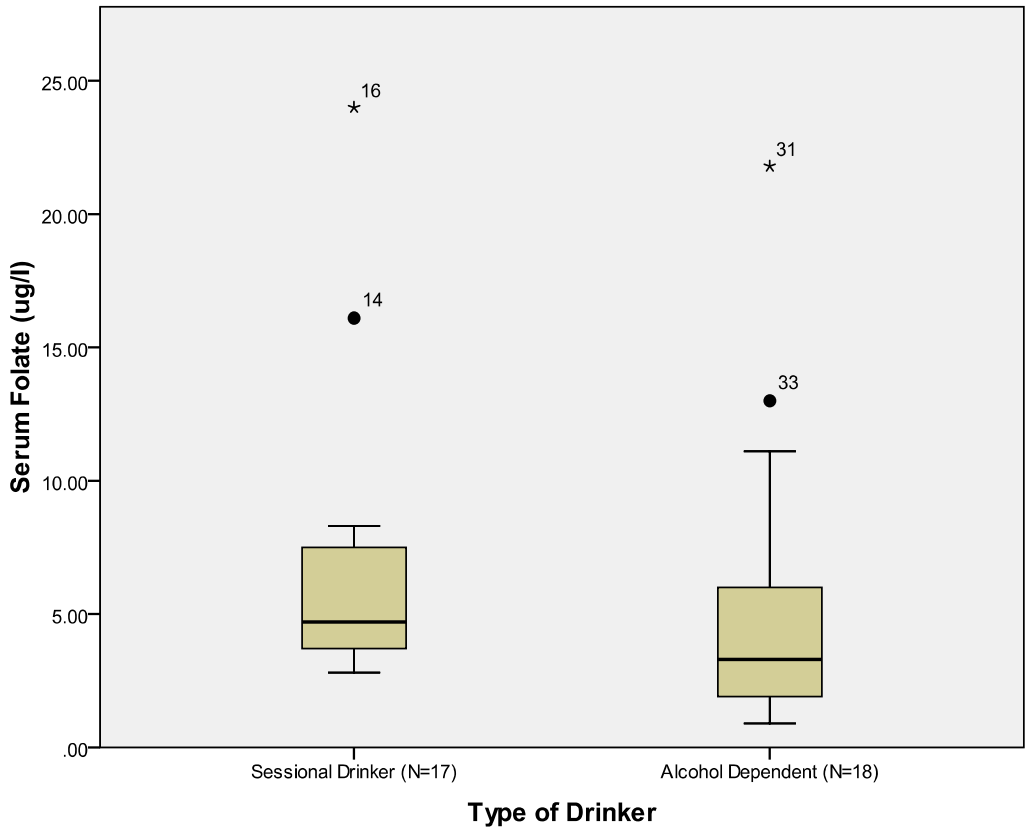

Figure 3.50: Box plots of serum folate in a sample of sessional drinkers (measured at baseline) compared to alcohol dependent patients (measured on day 1) *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

3.10.4 Serum Vitamin B_{12} Levels within Healthy Individuals (HI) who Consume Alcohol in a Range of Patterns

The vitamin B_{12} concentration identified in each of the alcohol drinking pattern groups is presented in figure 3.51. There was no significant ($p=0.139$) change in serum vitamin B_{12} levels at each pre-defined study time-point. In the abstainers group the vitamin B_{12} levels significantly increased ($p=0.01$) throughout the duration of the study. However there was no significant change in vitamin B_{12} for the other alcohol drinking groups.

Since the remethylation of homocysteine to methionine is dependent on vitamin B_{12} as a cofactor, a correlation was used to determine if there was an association between serum vitamin B_{12} and plasma homocysteine at baseline measurement and no correlation was evident. There was also no correlation between plasma homocysteine and serum vitamin B_{12} at the subsequent three and six month point time-points.

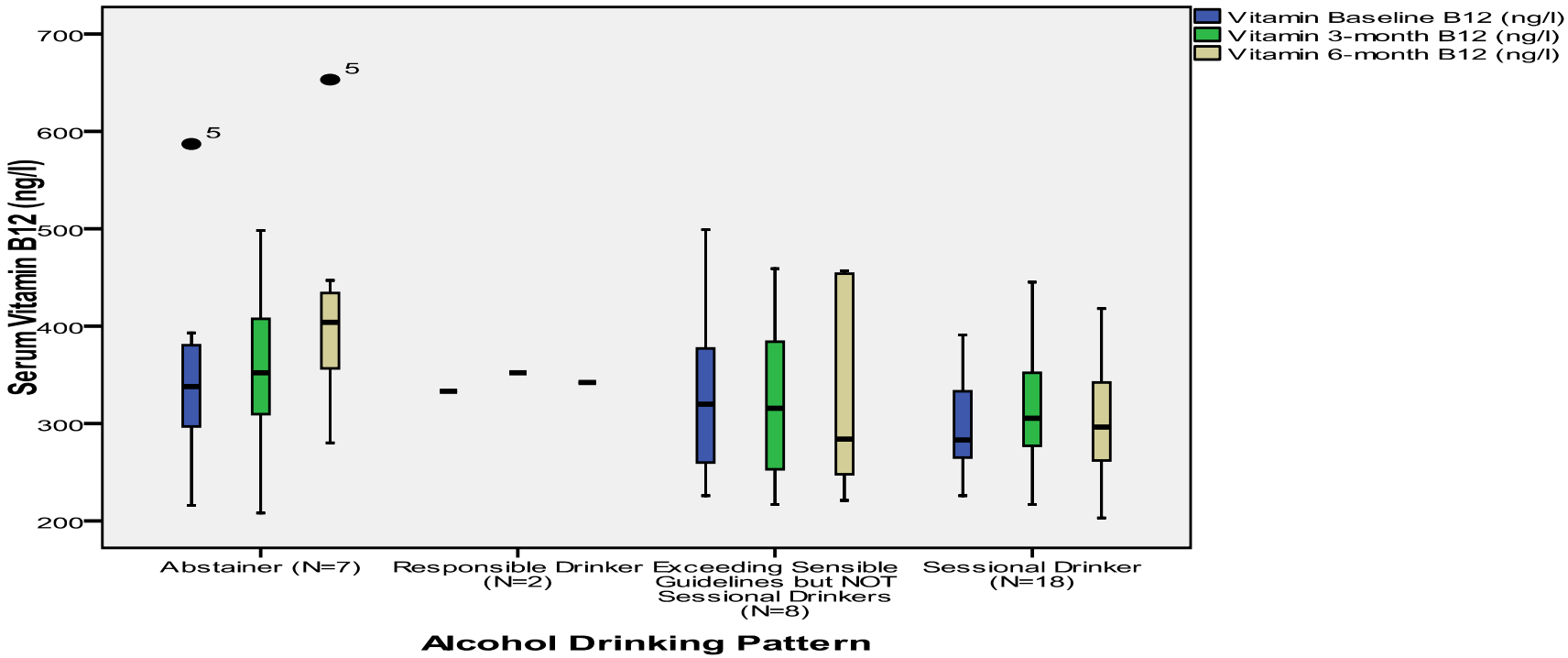

Figure 3.51: Box plots of serum Vitamin B_{12} (ng/l) for HI study (N=35) categorised according to alcohol drinking pattern over duration of study *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

At baseline, 91.4% (N=32) of the sample had serum levels within the clinical reference range (200-900 ng/l) for vitamin B_{12} levels. The remaining 8.6% of the sample (N=3) had vitamin B_{12} levels below the minimum clinical reference level of 200 ng/l in serum. These participants were female (see figure 3.52). All, had different drinking patterns, none were abstainers. One participant also had a serum folate level at baseline below the clinical reference range of 5 µg/l.

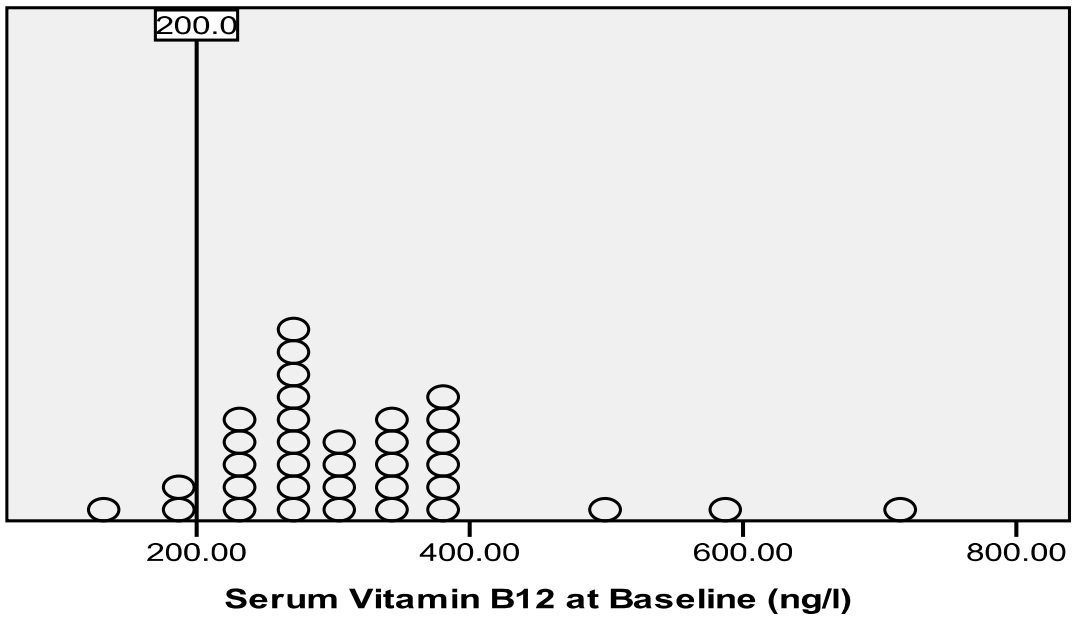

Figure 3.52: Dot plot of Vitamin B_{12} levels in HI Study sample (N=35), in relation to clinical reference range. The vertical reference line represents the minimum (200 ng/l) clinical reference range for vitamin B_{12}.

3.10.5 Serum Vitamin B_{12} Levels within Alcohol Dependent Individuals (ADI)

The concentrations of vitamin B_{12} identified in the ADI study sample at the predetermined study time-points are shown in figure 3.53. Vitamin B_{12} serum levels did not change significantly over the first 72 hours of alcohol detoxification (p=0.119).

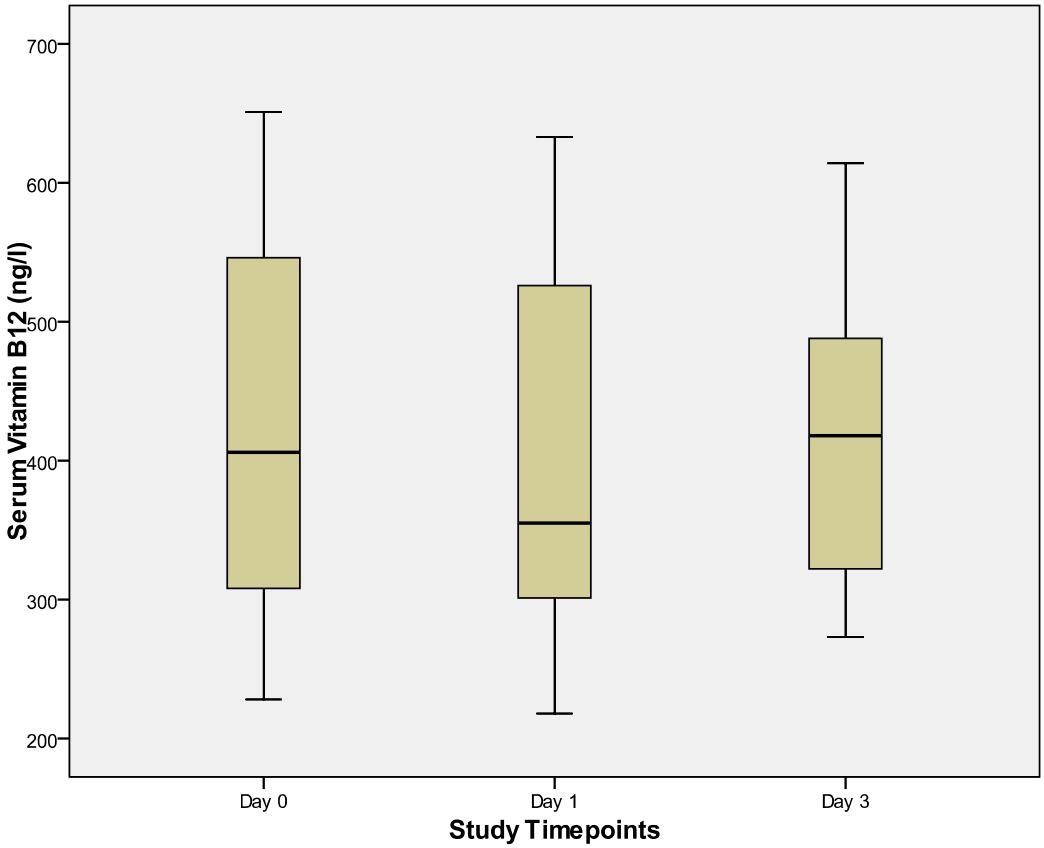

Figure 3.53: Box plots of vitamin B_{12} in serum (ng/l) for ADI study sample at three predetermined time-points *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

As illustrated within figure 3.53 all participants' serum vitamin B_{12} levels were within the clinical reference range of 200-900 ng/l at each time-point. There was no significant correlation between volume of daily alcohol consumption and vitamin B_{12} levels at each time-point in the ADI study sample. There was a significant inverse correlation between serum vitamin B_{12} measured on day 1 and homocysteine measured on day 1 (p=0.042, R=-0.594) (see figure 3.54). The statistical test was repeated for measurements on day 3, where the result was more significant and the correlation coefficient increased (p=0.001, R=-0.864), which is highlighted in figure 3.55.

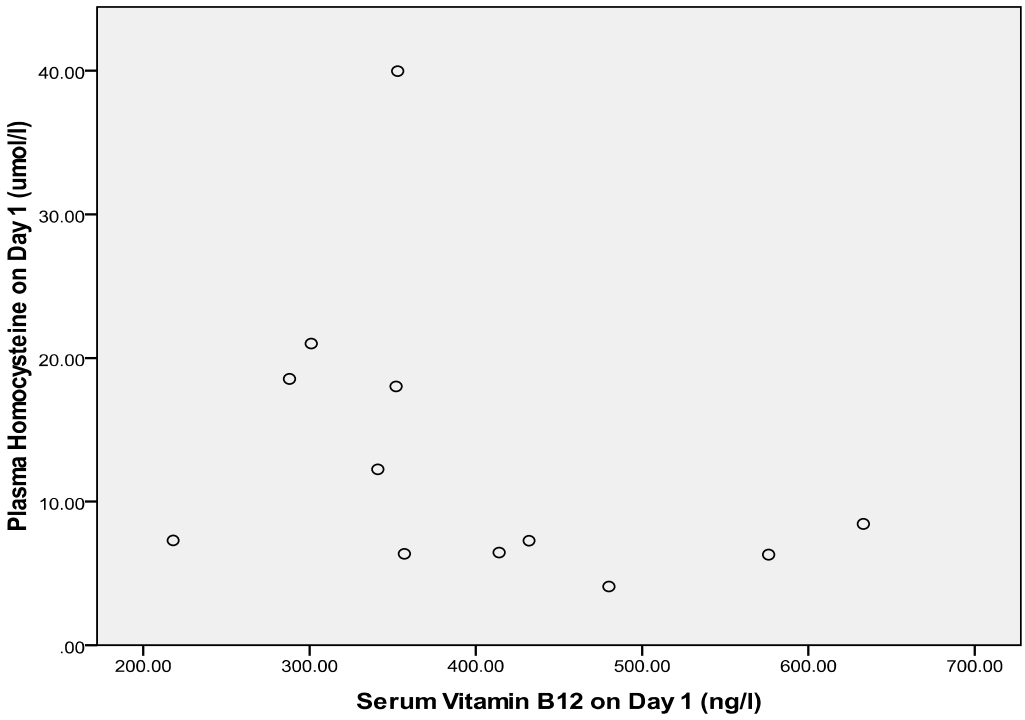

Figure 3.54: Scatter plot of serum vitamin B_{12} and plasma homocysteine on day 1 in a sample of ADI participants (p=0.042, R=-0.594 Spearman's test).

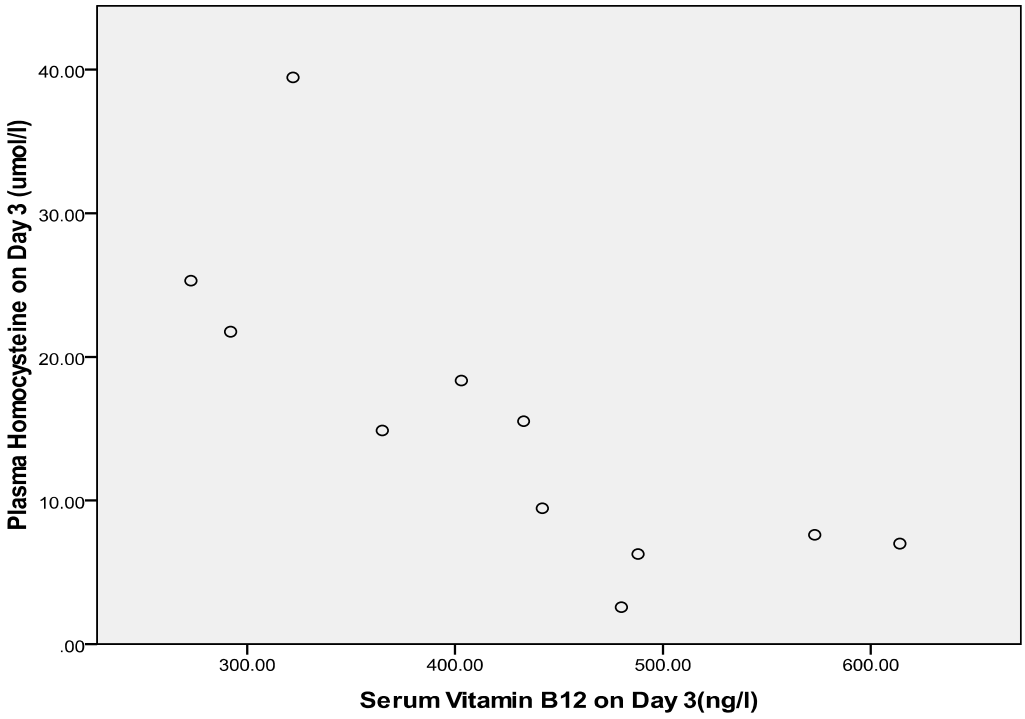

Figure 3.55: Scatter plot of serum vitamin B_{12} and plasma homocysteine on day 3 in a sample of ADI participants (p=0.001, R=-0.864 Spearman's test).

3.10.6 Serum Vitamin B_{12} Levels within Drinkers Combined Sample (HI and ADI)

The range of vitamin B_{12} levels in serum within a sample of drinkers (N=44) highlighted that 5.13% of the study sample was below the minimum clinical reference range. A Spearman's two tailed correlation test showed there was no significant association between plasma homocysteine and serum vitamin B_{12} within this study sample (p=0.165, R=-0.230). There was a significant correlation (p=0.004) between alcohol consumption per day and vitamin B_{12} status in the combined sample (N=44) who consumed alcohol in a range of patterns (figure 3.56).

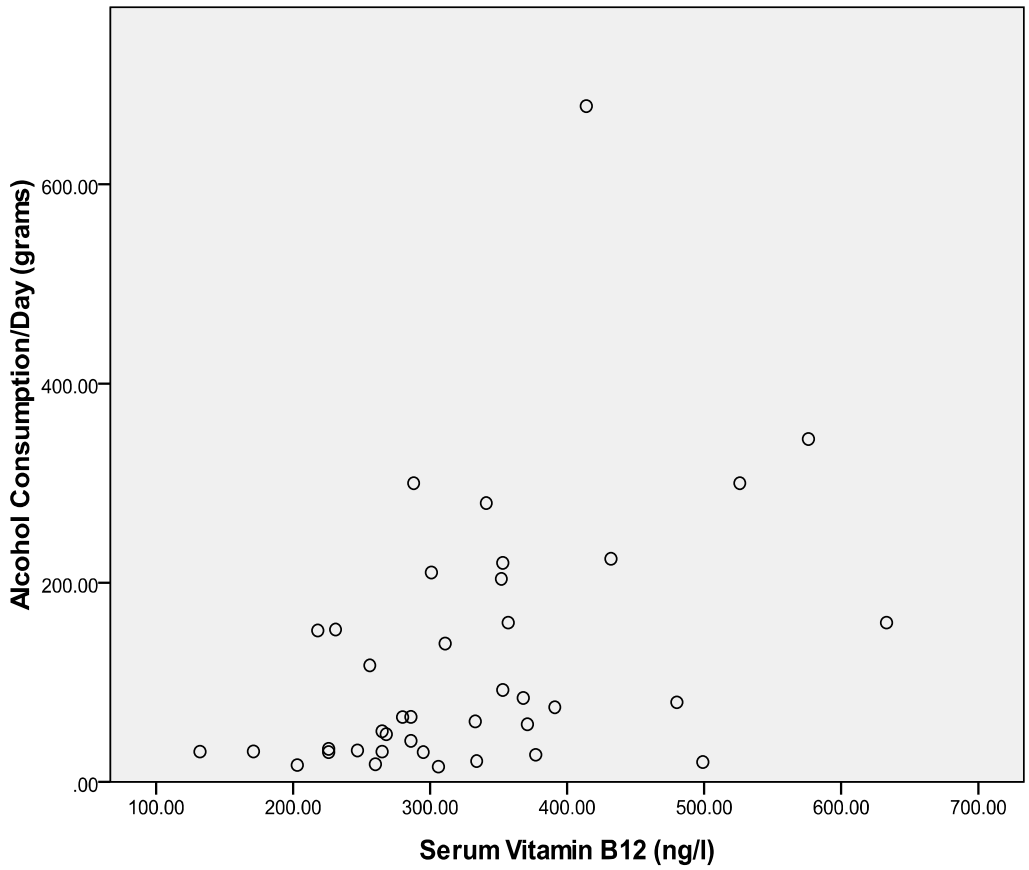

Figure 3.56: Scatter plot of serum vitamin B_{12} in serum (ng/l) in drinkers study sample in relation to alcohol consumed per day (g). (p=0.004, R=0.449 Spearman's test)

3.10.7 A Comparison Serum Vitamin B_{12} Levels within Sessional Drinkers and Alcohol Dependent Individuals

The median serum vitamin B_{12} level was higher in the alcohol dependent patient group compare with the sessional drinkers; 357 ng/l and 280 ng/l respectively (figure 3.57). Both these medians are within the clinical reference range for vitamin B_{12} of 200-900 ng/l. Within the sessional drinkers group there was one outlier, which was outwith the range of the box plot, and represented a value below the minimum clinical reference range of 132 ng/l. There was a significant (p=0.004) difference between the levels of vitamin B_{12} in the serum of sessional drinkers compared to that of alcohol dependent patients. This result suggests that vitamin B_{12} is significantly lower in sessional drinkers compared to alcohol dependent patients.

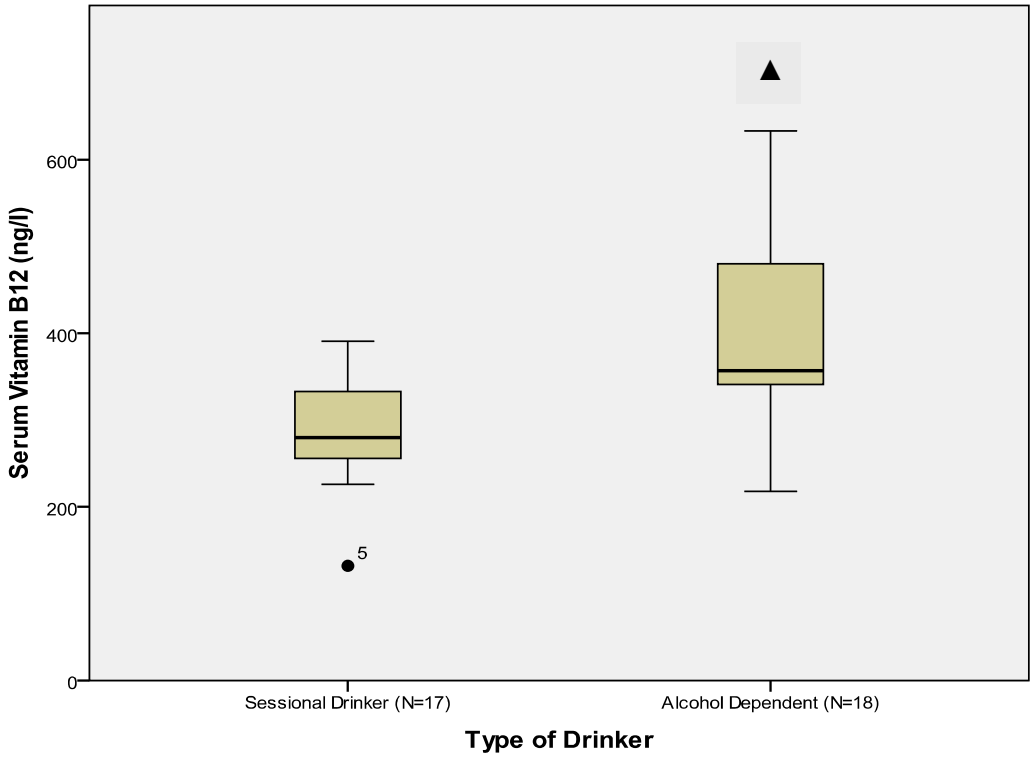

Figure 3.57: Box plots of serum vitamin B_{12} in a sample of sessional drinkers compared to alcohol dependent patients (▲p=0.004, Mann-Whitney test) *(Box plots represents the following: solid bar in box (median 50th percentile), top of box (75th percentile (upper quartile), bottom of box 25th percentile (lower quartile), top whisker (largest value which is not an outlier or extreme score), bottom whisker (smallest value which is not an outlier or extreme score). ● = Outlier (More than 1.5 box lengths above or below the box). * = Extreme case (more than 3 box lengths above or below the box.*

3.10.8: A Comparison of Biological Laboratory Analysis and Diet Diary Analysis for Folate and Vitamin B_{12}

Dietary nutrients including vitamin B_{12} and folate can be determined using either biological samples or through analysis of diet diaries.

3.10.8.1 Folate

For eight participants, folate was estimated both by biological laboratory analysis and through recorded dietary intake. No significant ($p=0.693$, $R=0.167$) association was detected between laboratory biological sample analysis and diet diary folate analysis.

3.10.8.2 Vitamin B_{12}

For eight participants, serum vitamin B_{12} was estimated in biological laboratory analysis and also from participant's dietary diary recall. No significant ($p=0.289$, $R=-0.429$) association was detected between laboratory biological sample analysis and diet diary vitamin B_{12} analysis.

3.11 Stability Study

Since the study design of both the HI study and ADI study required the storage of biological samples for periods of up to two years prior to analysis, it was essential to investigate the stability of the biomarkers under these conditions. Pooled serum and plasma samples were used to conduct the stability study and were subjected to the following conditions as described shown in table 3.16.

Table 3.16: Sample group freezing and thawing conditions for biological samples.

Group No.	Storage Condition
1	Spiked* samples at constant -80 ℃ (N=10).
2	Spiked* samples stored at a constant temperature (-80 ℃) but subjected to 3 occasions of thawing and refreezing (N=10)
3	Samples at constant -80 ℃ (N=10)
4	Samples stored at a constant temperature (-80 ℃) but subjected to 3 occasions of thawing and refreezing (N=10)

*Known concentration of homocysteine, folate, vitamin B_{12} and CDT standard.

3.11.1 Plasma Homocysteine

Table 3.17 describes the levels of plasma homocysteine found in each group and the percentage coefficient of variation for each group of test samples exposed to different storage temperatures, and thawing conditions.

Table 3.17: Plasma Homocysteine (µmol/l) within stability study groups subjected to different conditions (N=10)

	Group 1	Group 2	Group 3	Group 4
Median, Mean (SD; Range)	8.1, 8.4 (2.8, 5.2-13.4)	11.9, 12.1 (2.1, 9.0, 16.6)	1.3, 1.3 (0.3, 0.8-1.6)	5.2, 5.6 (1.3, 4.7-9.2)
%CV	33.2	17.3	24.4	23.8

There was a significant difference (p=0.005) between the plasma homocysteine levels detected within the group one and two samples. A significant difference (p=0.005) was also found between the samples stored according to the conditions of groups three and four. The samples in group one and group two were spiked with synthetic homocysteine, as described in the methods chapter. The concentration of the spike was 7.4 nmol/10 µl. The recovery of the spike in the samples stored in groups one and two is reported in table 3.18. The concentration of the mean spiked samples, mean non-spiked samples and percentage recovery of homocysteine are reported.

Table 3.18: Mean plasma homocysteine (µmol/l) levels in group one and two in relation to spike recovery

	Plasma Samples	Plasma Samples
With Spike (µmol/l)	8.4 (group 1)	12.1 (group 2)
Without Spike (µmol/l)	1.3 (group 3)	5.6 (group 4)
Recovery (%)	92.0	86.0

Plasma homocysteine concentrations must be directly compared to the storage conditions of each group. Whereby group one is compared to group three and group two compared to group four, as these samples were kept under the same storage conditions. The mean plasma homocysteine levels for groups one and three was 8.4 µmol/l and 1.3 µmol/l respectively, with a recovery of 92.0% of the synthetic homocysteine spike within the group 1 samples. The mean plasma homocysteine levels for groups two and four was 12.1 µmol/l and 5.6 µmol/l respectively, with a recovery of 86.0% in the plasma samples stored within group two and spiked with synthetic homocysteine.

The HPLC batch analysis of the samples was undertaken as two separate runs, with samples in groups one and three analysed together and samples in group two and four together. After investigating each of the batch calibrations it was identified that there was a 20% inter-assay variation, which explains the difference in the values for each group, meaning a direct comparison of groups one and three and groups two and four is not feasible. However the recovery of the spike does suggest that the samples were not affected by the repeated freezing and thawing. The explanation for the difference in the inter-assay variation could be due to HPLC column degradation and this requires more investigation.

3.11.2 Serum Folate

Table 3.19 describes the levels of serum folate found in each group and the percentage coefficient of variation found in each stability group which were exposed to different temperature and thawing conditions. The results for groups one and two were above the maximum assay detection limit of 20 µg/l, due to the spiking of the sample with folic acid, meaning no results could be reported.

Table 3.19: Serum Folate (µg/l) within groups subjected to different conditions (N=10)

	Group 3	Group 4
Median, Mean (SD; Range)	12.6, 12.5 (0.2, 12.1-12.9)	12.6, 12.5 (0.5, 11.2-13.0)
%CV	1.9	4.1

There was no significant difference (p=0.858) between the samples stored according to the conditions of groups three and four. The coefficient of variation between each group was below 10%, indicating that there was no variation between samples. Repeated thawing and refreezing of serum samples had no effect on serum folate estimation.

3.11.3 Vitamin B_{12}

Table 3.20 describes the levels of serum vitamin B_{12} found in each group and the percentage coefficient of variation found in each stability group which were exposed to different storage temperature and thawing conditions. The spiked samples in groups one and two, elevated the levels of vitamin B_{12} above the maximum assay detection limit of 900 ng/l, explaining the non-reporting of groups one and two results.

Table 3.20: Serum Vitamin B_{12} (ng/l) within groups subjected to different conditions (N=10)

	Group 3	Group 4
Median, Mean (SD; Range)	348.0, 368.1 (66.2, 320.0-545.0)	337.5, 341.40 (23.5, 315.0-388.0)
%CV	2.6	6.9

There was no significant difference (p=0.333) between the samples stored according to the storage conditions of group three and four. Therefore suggesting that repeated thawing and refreezing of the serum samples had no effect on vitamin B_{12} levels.

3.11.4 Carbohydrate Deficient Transferrin (CDT)

Table 3.21 describes the percentage coefficient of variation and %CDT concentrations found in each stability group, exposed to different temperature and thawing conditions. Only five samples from each group were analysed for CDT levels.

Table 3.21: %CDT in serum, within groups subjected to different conditions (N=5)

	Group 1	Group 2	Group 3	Group 4
Median, Mean (SD; Range)	1.5, 1.5 (0.1, 1.5-1.6)	1.73, 1.71 (0.1, 1.6-1.8)	1.92, 1.88 (0.2, 1.5-2.0)	1.9, 1.9 (0.2, 1.7-2.1)
%CV	4.6	4.1	10.6	8.3

There was a significant difference (p=0.042) between groups one and two. However there was no significant difference (p=0.893) between the samples stored under the conditions applicable to groups three and four. The percentage coefficient of variation between all groups was less than 10%, indicating a low variation between each sample within the groups. The statistical tests therefore indicted that repeated freezing and thawing did not effect serum samples and subsequent CDT analysis. The presence of the synthetic CDT spike in groups one and two, could have interfered with the N-Latex immunoassay resulting in the significant difference in the two groups, however this does require more investigation.

CHAPTER 4: DISCUSSION

4.1 Measurement of Alcohol consumption

4.1.1 Alcohol Consumption Analysis from Questionnaires and Diary Recall

The determination of alcohol consumption through the use of questionnaires and prospective diaries allowed for the categorisation of study participants into drinking patterns and also determined the accuracy of self-reported alcohol consumption.

The HI study questionnaire did highlight the finding that 77.7% (N=14) of HI study participants classified themselves as sessional drinkers. From diary recall, analysed using the AM method, 18 participants in the HI study were found to be sessionally drinking. However from the HI study questionnaire, four participants did not classify themselves as sessional drinkers, but were found to be sessional drinkers from their diary recall. This could suggest they were unaware of their true alcohol drinking pattern or they did not want to disclose this information. This finding suggests that diaries are providing more detailed alcohol consumption information in comparison to questionnaires. The HI study participants completed prospective diaries for a period of seven days which allowed for a detailed analysis of alcohol consumption, within a typical week. As the diaries were seven days long, it included a weekend, which has been shown in the literature to be more commonly associated with sessional drinking (Deas and Clark 2009). The use of the prospective diary reduced the need for memory recall, which can be affected especially if alcohol consumption is heavy, however the completion of the diary after heavy consumption can still induce error in recall.

The information provided by the ADI sample in the study questionnaire gave a key insight into the type of alcohol and volume of alcohol which was consumed in a dependent pattern, and as stated above, the range was very large, up to 600 g per day. It would have been valuable to ask ADI study participants to distinguish which type of wine they consume (e.g. red, white or rose) and also which type of cider they regularly consumed (e.g. white or fruit cider), as it has been described in the literature that homocysteine can be influenced by type of alcohol (Bleich et al. 2000c; Tsang et al. 2005). This additional information would have allowed a comparison of biomarker levels in relation to type of alcohol consumption and this type of question should be considered in future research.

The recording of alcohol consumption in questionnaires and diaries has been shown to be useful in both the HI and ADI studies and the published literature (MacAskill et al. 2008). ADI study participants were not asked to keep diaries as they were undergoing detoxification treatment and it was advised by the collaborating clinician that diaries would not be maintained successfully during this time. To truly quantify the consumption of alcohol it is important to use the most definitive method to analyse the consumption levels recorded, as the method of analysis can determine drinking pattern and therefore theorise about associated risk. However the use of a less detailed consumption method analysis can wrongly categorise an individual into the incorrect alcohol drinking pattern. This was demonstrated when alcohol diaries were analysed using the AM, WD and ONS methods.

Alcohol consumption was underestimated by both the WinDiets software method and the ONS guidelines scale, however there was a significant difference ($p=0.001$) in the alcohol analysis results, when comparing the ONS method to the AM method. The ONS guidelines method significantly underestimated alcohol consumption within a sample of HI study participants. The results also showed that the WD method and ONS method of analysis miscategorised individuals drinking patterns by 11% and 55% respectively and was significantly ($p=0.0001$) different to the participants categorised using the AM method. Further analysis of two participants who would have been miscategorised using the WD and ONS methods as non-sessional drinkers, exhibited plasma homocysteine levels above the maximum clinical reference range of greater than 15 µmol/l, suggesting potential CVD risk, due to elevated plasma homocysteine. It could be argued that WinDiets dietary analysis software is not primarily used to analyse alcohol consumption. However this suggests that specific alcohol analysis software should be made available and updated regularly with new alcohol brands being made available and sold to the general public. The AM method is a simple method, however it can be time consuming but in comparison to other alcohol consumption analysis methods it did prove more accurate, as it takes into consideration beverage brand, volume and %ABV. It is also important to note that although the AM method takes into consideration important alcohol consumption information, it is still dependent on the honesty and recall of the participant.

The present findings indicate that in studies dependent on participant recall of alcohol intake, a failure to account for individual drink sizes and specific drink alcohol content, may seriously underestimate intake and the proportion of drinkers exceeding responsible guidelines. This is clearly true of the ONS method, whereby over 35% of participants were miscategorised. Investigations which explore the relationship between alcohol intake and CVD disease risk will benefit from measures which improve the accuracy of the quantification of alcohol intake. In turn the evidence base of health guidelines will likely be improved (see appendix 4) (Murdoch et al. 2009).

From the investigation into the grams of alcohol consumed per day within the HI study a fourth group was identified, which was named as "exceeding responsible guidelines but not sessional drinkers". This group of HI study participants consumed alcohol out with the UK Department of Health's guidelines of 16-24 g (female) and 24-47 g (male) per day, but did not exceed the sessional drinking definition of greater than 48 g (female) and 64 g (male). Within the HI study sample there were eight participants who were included within this alcohol drinking pattern category. This finding of a fourth alcohol drinking pattern, which is not defined in the literature, shows that alcohol drinking patterns can not be easily defined without quantitatively investigating an individual's alcohol consumption.

Over the 6-month period of the HI study the alcohol drinking patterns of the study participants did change in relation to their consumption, whereby 37.1% (N=13) of the sample changed their alcohol drinking pattern at the 3-month time point, and 34.3% (N=12) of the sample changed their alcohol drinking pattern at the 6 month time point. The finding highlights the fact that drinking patterns do fluctuate over time and are subject to change. The total HI study sample median alcohol consumption during drinking days did decrease over the study duration, however this was not statistically significant (p=0.910). The effect of monitoring an individual's drinking patterns is also evident whereby over the six month period of the study the number of abstaining individuals increased, however this figure did include the original abstainers, but was increased by those participants who did not consume alcohol on the subsequent week at the time of monitoring.

The consumption data collected allowed for a comparison of the grams of alcohol consumed by both an alcohol dependent individual and a healthy individual who consumed alcohol in a range of patterns. The result indicates that sessional drinkers are potentially exposing themselves to the same level of alcohol intoxication and health risks as an individual who is dependent, however the level of potential health risk would be determined by the number of times sessional drinking is being undertaken, e.g. every weekend. It is also important to clarify that alcohol consumption determined from questionnaires and diaries and compared to a biological variable, does pose a potential serious error, as alcohol consumption determined from questionnaires and diaries is dependent on memory recall. Under heavy alcohol consumption, accuracy of memory recall is reduced, therefore evoking a potentially serious error.

4.2. Carbohydrate Deficient Transferrin (CDT) as a Biomarker of Alcohol Consumption

In an attempt to investigate the utility of Carbohydrate Deficient Transferrin (CDT) as a biomarker, analysis of serum samples was undertaken for both the HI and ADI studies. Within the ADI study, CDT levels were shown to significantly decrease ($p=0.017$) from day 1 to day 3, indicating that CDT was affected by alcohol consumption and subsequent alcohol detoxification. Within this group 69.2% (N=18), had a %CDT of greater than 2.6% which is a positive result using the N-Latex immunoassay on day 1. From self report, median daily alcohol consumption prior to entering detoxification treatment was 222 g and the range of consumption over the entire sample was 80-678.6 g. This range of alcohol consumption would be more than sufficient to elevate CDT levels in each of the ADI participants. Published literature (Jeppsson et al. 2007) has shown that CDT is a sensitive biomarker for alcohol intake exceeding 50 grams per day.

All sessional drinkers were shown to have a positive CDT result of greater than 2.6%. In specifically investigating the results from the CDT analysis of sessional drinkers a linear relationship ($p=0.01$, $R=0.98$) was identified between the number of drinking days within a 7 day period and a %CDT positive result. This suggests that the number of days within a week, whereby sessional drinking is undertaken, increases the level of serum CDT, resulting in a positive diagnostic result. A search

of the literature has identified that the linear correlation coefficient stated above, where the association between the number of drinking days and the %CDT result using the N-Latex immunoassay has not been identified before, in a sample of self-reporting healthy individuals who undertake sessional drinking. Due to the expensive nature of the N-Latex immunoassay, only a specific number of samples from the HI study could be analysed and as the published literature lacked research into the use of CDT as a biomarker within non-alcohol dependent individuals, serum samples were selected from the study, which were from participants who consumed alcohol in a sessional pattern or above the responsible alcohol consumption guidelines. To act as a control, serum samples from self-reported abstainers were also included for analysis, and showed no elevation, as would have been expected.

As stated previously the results of the HI and ADI studies have shown that healthy individuals who consume alcohol in a sessional pattern can consume the same amount of alcohol (grams) as an alcohol dependent individual on a given day. This has allowed for a direct comparison of sessional drinkers to alcohol dependent patients for CDT levels, which showed that both study groups had median CDT values which were positive (>2.6%), although significantly higher ($p=0.030$) in the ADI group. This higher result could be explained by the time-point at which blood was taken for both study samples. The serum samples taken for the ADI group were 24 hours after beginning detoxification treatment, compared with day eight of the sessional drinker's blood sample. Theoretically the sessional drinkers could have consumed alcohol earlier in the seven day monitoring period and had more alcohol free days before blood sampling, producing a lower CDT results. The rationale for the previous statement can be justified directly from the literature (Helander 2007), as the half-life of CDT is seven days, whereby if a HI participant had consumed alcohol on day one of their monitoring week and had six alcohol free days, this alcohol free period, would be a sufficient time period to allow CDT levels to potentially return to normal levels (e.g. a negative results of less than 2.6% in serum). However, the median and mean %CDT measurements in the sessional drinkers group was positive (>2.6%), suggesting that this biomarker can be used to monitor alcohol consumption, in a sample of sessional drinkers. As widely stated in the literature, the consumption of greater than 50 g of alcohol for a two week period is sufficient to elevate CDT levels. The results of the CDT analysis from sessional drinker's serum samples, suggests that sessional drinkers were consuming 50 g or

greater of alcohol, daily, for a two week period before blood sampling. However the HI study monitoring period was a 7 day period, and a suggestion of this work, could be to increase the monitoring period to 14 days, which would give a more definitive answer in terms of the amount of alcohol required to elevate CDT levels in sessional drinkers. It would also allow the determination of exactly how many drinking days is required to elevate CDT levels, with a view to potentially finding that it is less than two weeks of daily alcohol consumption to induce an elevation, as stated in the literature.

There was no significant ($p=0.893$) difference between the CDT levels found in the serum samples which were stored at either constant -80 ºC or subjected to multiple thawing and refreezing. The co-efficient of variation between each group was less than 10%, indicating no great variability between the samples. From the work carried out within this quality control study for the stability of CDT, it can be suggested that repeated freezing and thawing of the serum sample does not affect the analysis of CDT using the N-Latex immunoassay. The implications of this are, a recommendation can be suggested to laboratory researchers investigating CDT using the N-Latex immunoassay, that serum samples can be stored at -80 ℃ and repeatedly thawed and refrozen without effecting the subsequent analysis and results.

The results from the stability study did show a significant ($p=0.042$) difference between samples which were spiked with a synthetic CDT reagent and stored either at -80 ºC or multiple occurrences of thawing and refreezing. It is important to note that these samples were spiked with a synthetic CDT reagent which is used for calibrating an HPLC system for CDT analysis and this could have interfered with the N-Latex immunoassay which was used. The synthetic CDT reagent was purchased from Bio-Rad and is specially prepared human serum, with additional synthetic CDT added and is normally used to calibrate an HPLC system prior to analytical runs. The effect of this reagent being analysed by the N-Latex immunoassay method is unknown and from the stability study results, it is suggested that the N-Latex method does not detect the synthetic CDT. The multiple temperature changes, potential effects on this synthetic CDT reagent has not been established and could be the cause for the difference in the groups, however the non-spiked groups did show no

significant difference in CDT levels, thereby concluding that it was the artificial CDT which resulted in the difference between the groups.

4.2.1 Method Development for the Detection of Carbohydrate-Deficient Transferrin (CDT) in Serum Using HPLC

There are several methods which can be utilised to measures CDT in serum and include: HPLC, immunoassay and capillary electrophoresis. The current reference method for CDT analysis is HPLC with ultraviolet detection (UV). However there is work being undertaken to validate a LC-MS method for the detection of CDT, which will provide more sensitive analysis (Oberrauch et al. 2008). The current reference method published by Helander et al. (2003), was developed as part of this thesis and will be described within the following section.

CDT has been described in the published literature as a stable and reproducible biomarker for the detection of alcohol consumption. It has mainly been used to monitor abstaining within individuals who are dependent upon alcohol and are undergoing detoxification treatment. According to the International Federation of Clinical Chemistry Working Group on CDT (IFCC-WG-CDT) the current methodology used for the detection of CDT in human serum is by HPLC using UV detection and is outlined in the following published paper (Helander et al. 2003).

This work was unsuccessful, in validating a functioning HPLC CDT assay, using an HPLC system at QMU and a separate HPLC system at the University of Edinburgh. During the method development at both institutions, careful preparation was carried out to make sure the pH and ionic strength of the mobile phases were exactly as stated in the paper by Helander et al. (2003), as this can have an effect on ion-exchange HPLC. The major difference between the work which was carried out during the method development for this assay and the method outlined within the published paper was the manufacturer of the HPLC column, which was used for the separation of the CDT glycoforms. The column used in the method development for this technique was an ion-exchange column manufactured by Phenomenex, whereas within the method paper published by Helander et al. (2003), the column used by the authors was manufactured by GE Healthcare.

The HPLC columns manufactured by Phenomenex and GE Healthcare are ion-exchange columns, which are suitable for separation of the CDT glycoforms. However as separation of the individual glycoforms was not successful, using the Phenomenex branded column, it would have been good practice to carry out the HPLC analysis using the GE Healthcare column, which was the chosen column within the published method paper. Unfortunately this could not be carried out due to the cost of the GE Healthcare column. The manufacturer of the HPLC column was the only major change in the method development for the detection of CDT, it can be suggested that this was the cause of the unsuccessful separation of the CDT glycoforms. The functionality of both the Phenomenex column and the GE Healthcare column was to act as an ion-exchange separation device, whereby the glycoforms of CDT would be separated within the column and eluted at the appropriate retention time. Each differently manufactured column can have different chemical resins, which can alter the binding of the CDT glycoforms to the column and thus alter the elution and retention times, however both the GE Healthcare and Phenomenex columns were the same type of ion-exchange column and variation of chemical resins should not have altered the separation of CDT, to a point where separation was not possible. The resin used within the Phenomenex Clarity Wax column was high purity silica bound to a cross-linked polyamide polymer and the resin used within the GE Healthcare SOURCE 15Q PE 4.6/100 column was polystyrene/divinyl benzene. The resin within the GE healthcare column, is a more commonly used resin for ion-exchange chromatography, however the Phenomenex column was chosen as it is a direct equivalent to the GE Healthcare column. Furthermore due to budget restraints the Phenomenex column was chosen, due to it lower purchase price. As the Phenomenex column is a direct equivalent to the GE Healthcare column, it was not predicated to cause non-detection of the CDT glycoforms, however as the method development work did not produce a working HPLC assay, it must be considered that the different type of column impacted on the assay development and functionality.

The method development results for CDT did show that using the Phenomenex column an identifiable transferrin peak at approximately 15 minutes eluted, however further mass spectrometry of separated fractions, showed that the peak at 15 minutes included all transferrin glycoforms but did not identify the individual glycoforms, which give a CDT concentration. This result does suggest that although

the Phenomenex column did separate and elute transferrin glycoforms from the serum sample, it wasn't sensitive enough to separate out each glycoform into identifiable peaks. This could be the reason why the method development did not produce a working method as outlined in Helander et al. (2003). Another point to consider when investigating why the method did not work was the lack of detail within the Helander et al. (2003) published paper, from which the method development was based on. The published paper could have lacked specific detail on the HPLC system, sample preparation or mobile phase gradients, which would have contributed to the lack of success in developing a method which would suitably separate each CDT glycoform.

Further work would have to be conducted, with a view of comparing the method development conducted in this thesis and the use of the exact column described within the peer-reviewed article (Helander et al. 2003). It is also important to note that the HPLC method for the detection of CDT in human serum is an intermediate method until a fully validated liquid chromatography-mass spectroscopy (LC-MS) method has been approved by the IFCC-WG-CDT (Jeppsson et al. 2007). The LC-MS method, which is more sensitive and accurate in comparison to HPLC, is currently under development, and work relating to the method has been published in the literature (Oberrauch et al. 2008).

The N-Latex immunoassay showed sensitivity and specificity to samples from each study, whereby identifying that CDT can be used as a biomarker to detect alcohol consumption in the form of sessional drinking as well as alcohol dependent drinking. This has shown that the N-Latex immunoassay, although not as sensitive as HPLC or LC-MS techniques does have potential to identify participants who are consuming alcohol in sessional and dependent patterns. The use of the N-Latex immunoassay to confirm hazardous alcohol consumption in a sample of sessional drinkers has not been published in the literature.

4.3 Plasma Levels of the CVD Risk Biomarker Homocysteine within Individuals with a Range of Alcohol Consumption Patterns

Within the HI group of drinkers, some individuals (N=8) exhibited plasma homocysteine levels out-with the normal clinical reference range of 5-15 µmol/l; two of those participants exceeded responsible drinking guidelines and four were sessional drinkers. However within the entire HI group, there was no correlation between the mean alcohol consumption during drinking days for the 7 day period of monitoring and plasma homocysteine levels. This was also true when the analysis was repeated for each of the time-points within the HI study.

Within sessional drinkers (N=17), the median plasma homocysteine levels were shown to be higher than that of abstaining individuals and four participants had homocysteine levels which were out with the normal clinical reference range of 5-15 µmol/l. There was no significant association between the mean alcohol consumption per drinking days and plasma homocysteine levels in sessional drinkers. A larger sample size could have clarified if an association was evident or not.

Further investigation of the drinkers in the HI study showed that the median plasma homocysteine levels increased for the sessional drinkers and responsible drinkers at each subsequent time-point during the study period, but this was not statistically significant. No change in median plasma homocysteine levels during the study was found in abstaining individuals. Unexpectedly the two responsible drinkers were shown to have plasma homocysteine levels which were higher than the sessional and exceeding responsible guideline drinkers. However this could simply be explained by the impact of factors unrelated to alcohol consumption. One of the responsible drinkers was positive for the mutant $MTHFR_{(C677T)}$ polymorphism which is associated with higher plasma homocysteine levels. The other responsible drinker was genotyped as wild-type heterozygote for the $MTHFR_{(C677T)}$ polymorphism.

In the ADI study, a number of participants (N=4) exhibited plasma homocysteine on day 1 which were above the clinical reference range. By day 3, 72 hours after alcohol detoxification treatment was initiated, the median plasma homocysteine level

was above 15 µmol/l, however this was not a significant increase (p=0.117) from day 1. There was a non-significant correlation between the self reported amount of daily alcohol consumed prior to detoxification and plasma homocysteine levels on day 1 (p=0.754, R=0.096) and day 3 (p=0.615, R=-0.142). Literature evidence would suggest the opposite; plasma homocysteine is likely to fall during this time period (Bleich et al. 2000 and Devika et al. 2008). The work by Bleich and co-workers showed a reduction in homocysteine from 33.6±25.2 µmol/l to 13.9±8.8 µmol/l in a 72 hour period from the beginning of detoxification treatment; however the paper by Bleich et al. 2000 did not state if this reduction was significant and no p-value was stated. Devika et al. (2008) found homocysteine significantly ($p<0.002$) reduced from 26.00±13.36 µmol/l to 14.31±5.7 µmol/l within eight weeks under the influence of supplementation with folic acid and vitamin B_{12}. As the study by Devika et al. (2008), was over a longer time period and involved vitamin-reducing supplementation, the study cannot be directly compared to the ADI study. However, one possible, but speculative explanation (given the low number of participants) for the discrepancy between the present findings and those of Bleich et al. (2000) is the potential impact of the type of alcohol has on homocysteine levels. In the ADI study the largest rise in the amino acid was detected in the two participants who consumed cider. The studies by Devika and Bleich were in Indian and German alcohol detoxification clinics, respectively. The particular type of drinks consumed by their participants is not reported.

It is important to state that the participants recruited to the ADI study were undergoing a medically supervised alcohol detoxification, which included the prescription of Chlordiazepoxide (benzodiazepine) to reduce the withdrawal side effects from alcohol. This was part of the protocol for this study and was similar to other studies of this design published in the literature (Bleich et al. 2000a; Bleich et al. 2000d; Cravo and Camilo 2000; Cravo et al. 1996). Known side-effects of the withdrawal from alcohol dependence include vomiting, tremors, seizures and headaches (Kumar et al. 2009). The use of Chlordiazepoxide was prescribed as a reducing regime over the course of the detoxification. All participants recruited to the ADI study were prescribed Chlordiazepoxide in a dose which ranged from 40 mg to 180 mg per day. There is no evidence published in the literature that suggests Chlordiazepoxide interacts with, or affects, plasma homocysteine levels.

A literature search provided one recent study, published in the last two years, which investigated the relationship between plasma homocysteine and alcohol consumption, but only investigated healthy male subjects (Gibson et al. 2008). (In the HI study only 34.3% (N=12) of the participants were male.) This randomised, cross-over study, was an intervention whereby participants were asked to consume either 24 g of vodka or red wine daily, for a two week period and plasma homocysteine was measured before and after the intervention. The amount of alcohol consumed (24 g; 3 UK units) was within the UK Department of Health guidelines for responsible daily consumption for males (UK Department of Health 1995). Results indicated that the consumption of 24 g of red wine for a 2 week period significantly ($p=0.03$) elevated plasma homocysteine levels compared to levels before the start of the intervention, however there was no significant increase in plasma homocysteine levels in the vodka drinking group. When both interventions were compared there was no significant difference ($p=0.57$) found between the levels of plasma homocysteine after the 2 week consumption of either red wine or vodka.

A similar study by Bleich et al. 2000c, who investigated alcohol consumption in a sample of male social drinkers (N=45) compared to abstainers (N=15) confirmed that red wine elevated homocysteine levels in serum, but also found an elevation in the serum of spirit drinkers. Additional literature suggests that red wine consumption reduces homocysteine levels due to the potential antioxidant effects of red wine (Tsang et al. 2005) in human subjects. The study by Tsang et al (2005), was a randomised control study, where study participants (N=12) were asked to consume 375 ml of 12% red wine (36 g; 4.5 UK units), daily for a two week period. The results from the Tsang et al. (2005) study shows that after the red wine intervention, plasma homocysteine levels reduced from 8.1 µmol/l to 7.8 µmol/l, but increased in the control group (N=8) from 9.8 µmol/l to 10.0 µmol/l. The consumption levels of red wine in the Tsang et al. (2005) study is above the UK Department of Health's recommended daily alcohol consumption guidelines of 3 units (female) and 4 units (male) per day, however the results show that there was a reduction in homocysteine levels after alcohol consumption, although this reduction was not statistically significant. It is important to note that the gender of the participants was not stated. The findings of the Tsang et al. (2005) study does contradict the findings of the Gibson study and ADI study, which further shows that there is

conflicting evidence to suggest that different types of alcohol vary in their influence on homocysteine levels and this warrants further investigation. The findings of Gibson et al (2008) (intervention study) and preliminary findings from the present work (self-reported consumption) do agree that the type of alcohol consumption can impact on plasma homocysteine levels. More work is required to investigate the exact type of alcohol and drinking patterns which promote a change in plasma homocysteine levels and thereby potentially increase the risk of cardiovascular disease.

The most recent study investigating plasma homocysteine levels in alcohol-dependent individuals was by Devika et al. 2008, who found a significant decrease ($p<0.002$) in serum homocysteine levels after an eight week alcohol detoxification. The eight week detoxification period, is longer in comparison to studies conducted by Bleich and co-workers and the present ADI study. This longer period for alcohol withdrawal would give more time for plasma homocysteine to return to lower levels. Due to the different time-scales of the studies by Devika et al. (2008), it is not appropriate to compare findings from this study to the findings in the ADI study and by Bleich et al. (2000b). Furthermore the study by Devika et al. (2008), also supplemented the participants with vitamin B_{12} and folic acid, which are known to decrease the circulating levels of homocysteine. Other studies by Bleich, Cravo and co-workers found similar results, however these studies did not supplement their study participants with folic acid and vitamin B_{12} and concluded that the simple withdrawal from alcohol did, on its own, reduce homocysteine levels (Bleich et al. 2000a; Bleich et al. 2000d; Cravo and Camilo 2000; Cravo et al. 1996).

The design of the ADI and HI studies permitted comparison of healthy individuals who undertake sessional drinking with alcohol-dependent individuals. Findings suggest that healthy individuals who undertake patterns of sessional drinking can consume the same volume of alcohol as an alcohol dependent individual inducing the potential health related risk factors associated with excess alcohol consumption, including a rise in plasma homocysteine, which have been well documented in the literature (Bleich et al. 2001; Trabetti 2008). A comparison of plasma homocysteine levels between sessional drinkers (median 7.20 µmol/l) and ADI individuals (median 7.89 µmol/l) showed no significant difference ($p=0.818$). However, in both groups, the median plasma homocysteine levels are within the clinical reference range for

homocysteine, which is internationally recognised as being 5-15 µmol/l in plasma (Antoniades et al. 2009; Ducros et al. 2002). The range of plasma homocysteine levels within each drinking pattern does extend above the clinical reference range, which suggests that hyperhomocysteinemia is present within sessional drinkers and alcohol dependent individuals. Both drinking patterns may be associated with a potential CVD risk. Therefore these individuals are at an increased risk of hyperhomocysteinemia, which includes oxidative stress and endothelium dysfunction, and can potentially lead to the development of cardiovascular disease (Trabetti 2008). More research is required with a larger sample size, to ascertain the effect different alcohol drinking patterns have on plasma homocysteine levels within a sample of individuals who consume alcohol in a range of patterns.

Since the present study design required the storage and subsequent batch analysis of homocysteine, it was essential that long term freezer storage did not jeopardise the stability of the amino acid and thereby invalidate laboratory analysis findings. Certainly literature evidence suggested that repeated freezing and thawing of plasma, has no effect on subsequent homocysteine analysis (Ducros et al. 2002). A significant difference was found between sample groups which were either spiked with synthetic homocysteine or non-spiked ($p=0.005$, $p=0.005$ respectively) and subjected to either constant storage temperatures or repeated thawing and refreezing. There was also a high percentage coefficient of variation within each group, above the threshold of 10% variation, which again highlights the evident effect of multiple freezing and thawing.

However, when the recovery of the artificial homocysteine spike was taken into consideration the median levels of each comparable group were similar. The batch analysis of the samples however did highlight that due to column degradation, there was a 20% variation in the inter-assay runs. The column degradation, reflected in the inter-assay variation, may be explained by the number of sample runs which had been analysed by the column, prior to the analysis of the stability samples. The column instructions advised that the column was sustainable for up to 700 analyses and this upper limit was almost reached during the analysis of all plasma homocysteine from the HI, ADI and stability study duplicate batch analyses. It would have been good laboratory practise to repeat the sample batch analyses on a new column, however financial restrictions prevented this. This prevented the

comparison of groups, due to the inter-assay variation, meaning a definitive conclusion of the effect of freezer storage on plasma homocysteine could not be reached. The functionality of the HPLC assay did show specificity in recovering the artificial homocysteine spike. Also the analysis of plasma samples was carried out using HPLC-ED, which produced consistent calibrations, with coefficients of determination of >0.99 and %CV of less that 5%, suggesting that the functionality of the assay method was reliable (Cummins 2005).

4.3.1 Method Development for the Detection of Homocysteine in Urine

Homocysteine was detected in urine but sensitivity was low and provided results for only 22.9% of HI study (N=35) urine samples and 38.9% of the total ADI study (N=18) urine samples. The results found do suggest that homocysteine is expressed at very low concentrations and the use of creatinine as a constant to measure homocysteine against**,** is required.

A reliable and accurate method for the detection of homocysteine in plasma or serum has been well established in the literature (Ducros et al. 2002; Houze et al. 2001). However the measurement of homocysteine in urine, would avoid the need for venepuncture, therefore reducing the risk of this procedure to both participants and phlebotomist. Also urine is a more convenient and a less invasive biological sample to collect. There was no significant correlation found between plasma homocysteine levels and homocysteine measured as a ratio with creatinine in urine. This finding suggests that plasma homocysteine is a superior method in analysing and determining homocysteine levels. However the urinary homocysteine assay was not fully validated and did require more work. It would be important to repeat this association with a fully validated urinary homocysteine assay and to test for any potential statistical association.

The measurement of daily alcohol consumption derived from diaries was also used to determine if there was an association between urinary homocysteine and alcohol consumption. This provided a large sample size (N=19). Using daily alcohol consumption recorded in grams, a significant inverse correlation (p=0.038, R=-0.480) was identified between self-reported alcohol consumption in grams (gained from questionnaires and diary recall) and urinary homocysteine expressed as a ratio

with creatinine. However an inverse correlation was not expected, as it would be anticipated for alcohol consumption to increase urinary homocysteine levels not induce a decrease. As the assay was not fully validated and lacked sensitivity, this could explain the findings. This association requires more investigation with an increased sample size and fully validated urinary homocysteine assay.

The urinary method by Thomson and Tucker 1986, was published several years ago using HPLC with electrochemical detection, which is the same technique used to measure plasma homocysteine. More recently a method paper by Kuśmierek and co-workers established a method for the simultaneous detection of cysteine, cysteinylglycine and homocysteine in urine using HPLC with ultraviolet (UV) detection (Kusmierek et al. 2006). However electrochemical detection is a more sensitive detection method to accompany HPLC, which produces more reliable results, in comparison to UV detection. The reliability of HPLC-ED has been established for homocysteine analysis (both in plasma and urine), through method development by numerous authors (Cummins 2005; Houze et al. 1999; Houze et al. 2001; Kusmierek et al. 2006). It is important to note that electrochemical detection is prone to stability problems, in comparison to ultraviolet detection. However the use of a gold-electrode has been established to improve sensitivity and counteract interference problems (Houze et al. 2001). This was the reason, why the method by Thomas and Tucker (1986) was used to develop a urinary homocysteine assay, instead of the method adopted by Kusmierek et al. (2006).

Details relating to another biomarker employed to estimate alcohol consumption is ethyl glucuronide, expressed relative to urinary creatinine levels has been previously published by Bergström et al. (2003). This paper investigated creatinine content in urine in a sample of drivers whose blood alcohol level was above the legal driving limit. The authors compared urinary creatinine levels to urinary levels of ethyl glucuronide, which is a direct ethanol metabolite. Urinary creatinine was measured using the Jaffe method, which is the same method employed within this thesis. The paper by Bergström et al. (2003) stated that creatinine concentration of less than 0.2 g/l in urine was a highly dilute specimen for detection of urinary biomarkers normalised using creatinine. This dilute concentration of urine would be evident as a result of an alcohol induced diuresis. This would occur during the early stages of alcohol consumption. In the later stages the urine is more likely to become

concentrated. Therefore Bergström et al. (2003) recommend that urine should be sampled at the second void, as opposed to the first void, to limit the effects of alcohol induced diuresis. When comparing the levels of urinary creatinine found within both the HI and ADI study sample, it was found that all the samples which were found to contain detectable homocysteine, had creatinine levels of greater than 0.2 g/l. This suggests that the remaining participant samples were simply too dilute (0.07-0.1 g/l creatinine), given the sensitivity of the urinary homocysteine assay. Alcohol dependent individuals may have provided a more concentrated urine sample at the beginning of alcohol detoxification, due to the dehydrating action of alcohol. The consumption of alcohol on urine production has been well documented in the literature (Lieber 2004). Consumption of alcohol, increases the metabolic rate of alcohol metabolism, by way of alcohol dehydrogenase, and the excretion of alcohol via the kidney. Urine production is increased by the inhibition of antidiuretic hormone or vasopressin, released from the pituitary gland (Swift and Davidson 1998). When antidiuretic hormone is inhibited this prevents the kidney from reabsorbing and conserving water, thereby increasing urine production (Swift and Davidson 1998). The urine produced during alcohol consumption is dilute and therefore biomarker measurement in urine must take into consideration the effect of alcohol on urine composition, which has been highlighted in the literature by Bergstom et al. (2003).

The work by Bergström and co-workers did produce a significant correlation ($p<0.001$, $R=0.62$) between the alcohol biomarker ethyl glucronide and urinary creatinine within the sample of intoxicated drivers (Bergström et al. 2003). However this correlation was more evident in the second urinary void sample, than the urine void directly after intoxication, as a more concentrated urine sample, produced a significant correlation of creatinine to ethyl glucuronide. Bergström and co-workers suggested expressing the alcohol biomarker ethyl glucuronide as a ratio with creatinine. The work by Bergström et al. (2003) has also highlighted that a dilute sample of less than 0.2 g/l of creatinine would produce less reliable results and dilute the levels of any potential biomarker which was being investigated, including ethyl glucuronide and the same theory can be applied to the investigation of homocysteine in urine. A dilute urine sample, due to the diuresis effects of acute alcohol consumption, would produce a urine sample which was not sufficiently concentrated, to detect suitable levels of any potential biomarker. Therefore when

using urine to determine an association between alcohol consumption and a biomarker (e.g. homocysteine), it is important to take into consideration the diluting effects of alcohol on urine, i.e. do not take a urine sample directly after intoxication.

In summary the detection of homocysteine in urine has been shown to be moderately successful in samples of healthy individuals and alcohol dependent individuals. However more work is required to develop the HPLC assay to detect the very low levels of homocysteine expressed in urine. The suggestion by Chwatko and Jakubowski to measure homocysteine-thiolactone, which is a thioester of homocysteine in urine needs further consideration (Chwatko and Jakubowski 2005). However, evidence in the published literature, suggests homocysteine-thiolactone has not been fully accepted as a biomarker for CVD risk and the measurement of total homocysteine is still preferred (Antoniades et al. 2009). The method published by Kusmierek and co-workers does show potential, and utilises HPLC with UV detection (which is more commonly available and less expensive to maintain). However a suggestion for further work would be to use a more powerful and sensitive technique such as liquid chromatography with mass spectrometry (LC-MS) (Ducros et al. 2002).

4.3.2 Prevalence of the $MTHFR_{(C677T)}$ Polymorphism and Effect on Plasma Homocysteine

A polymorphism is defined as a genetic mutation which is present in greater than 1% of the population. The mutant $MTHFR_{(C677T)}$ polymorphism was present in six participants within the HI study (N=35) but was not found in any participants in the ADI study (N=18). The expression of the mutant $MTHFR_{(C677T)}$ polymorphism in six participants within a total study sample of fifty three (N=53, HI and ADI studies) would be expected, as this is greater than one percent of the sample (11.3%). The exact prevalence of the mutant $MTHFR_{(C677T)}$ polymorphism in the Scottish population is yet to be determined. The presence of the mutant $MTHFR_{(C677T)}$ polymorphism is known to cause elevation of circulating plasma homocysteine levels, through the reduction in the activity of the MTHFR enzyme, whereby 5-MTHFR cannot act as a carbon donor during the remethylation of homocysteine to methionine (Fabris et al. 2009). Furthermore the presence of the mutant

$MTHFR_{(C677T)}$ polymorphism has also, therefore, been confirmed as a risk factor for cardiovascular disease due to consequent increase in homocysteine (Bennouar et al. 2007; Klerk et al. 2002).

From the results shown in chapter 3, there was no significant difference between the concentrations of plasma homocysteine found in the mutant homozygote $MTHFR_{(C677T)}$ polymorphism group compared to the wild-type homozygote or the heterozygote variants. The highest plasma values of homocysteine were in fact detected within participants who carried the wild-type homozygote genotype, which was not expected, however the median plasma homocysteine level within the mutant polymorphism group was higher and this finding is more consistent with what might be anticipated from literature results.

There have been a number of studies published in 2008 and 2009 which investigated the link between elevated plasma homocysteine levels and the presence of the $MTHFR_{(C677T)}$ polymorphism. The studies by; Alessio et al. (2008), Barbosa, et al. (2008), Grassi et al. (2008), Nagele et al. (2008) and Yang et al. (2008) all showed that the presence of the $MTHFR_{(C677T)}$ polymorphism affected homocysteine concentrations either in serum or plasma and was a major factor when investigating the cause of elevated homocysteine levels. When specifically investigating the participants who were found to carry the mutant $MTHFR_{(C677T)}$ polymorphism, the median plasma homocysteine range did increase over the 6-month study period compared to other genotype groups. This change was not statistically significant but may suggest that lifestyle factors and the presence of the mutant $MTHFR_{(C677T)}$ polymorphism are causing an effect on plasma homocysteine. Given the small sample size (N=5), further investigation employing a larger sample size is merited.

An analysis of the data for the HI study participants (N=5) who carried the $MTHFR_{(C677T)}$ mutation at baseline, found a significant linear correlation ($p<0.05$, $R=0.975$), between plasma homocysteine and mean alcohol consumption during drinking days. This association was not found in participants who were wild-type homozygote and heterozygote for the $MTHFR_{(C677T)}$ polymorphism. A comparison of the ratio of homocysteine to mean alcohol consumption during drinking days at baseline further highlighted the relationship between alcohol consumption and the

presence of the mutant $MTHFR_{(C677T)}$ polymorphism, where the ratio was higher in the group of participants who were genotyped for the mutant $MTHFR_{(C677T)}$ polymorphism. This highlights the potential for the individuals who carry the mutant $MTHFR_{(C677T)}$ polymorphism to increase their plasma homocysteine levels to 'at risk' levels, of above the maximum clinical reference cut-off value of 15 µmol/l, when consuming alcohol in situations which could be termed 'social drinking'. This finding has not been reported or investigated in the current published literature.

The relationship between the presence of the mutant $MTHFR_{(C677T)}$ polymorphism and the effect of alcohol consumption on plasma homocysteine levels requires more investigation within a larger sample of individuals who consume alcohol in a range of patterns, including dependency, to ascertain the effect of heavy alcohol consumption on plasma homocysteine levels in the presence of the $MTHFR_{(C677T)}$ polymorphism. There were no ADI participants found to carry the mutant $MTHFR_{(C677T)}$ polymorphism and the presence of this polymorphism within a sample of alcohol dependent individuals does require investigation.

4.4 Vitamin Co-factors of Homocysteine Metabolism

4.4.1 Serum Folate

Folate is an important co-factor involved in the normal remethylation of homocysteine to methionine. It is also well established in the literature that excessive alcohol consumption can reduce the bioavailability of folate *in vivo,* through a catabolic effect on the folate molecule.

The levels of serum folate and serum vitamin B_{12} associated with particular patterns of alcohol consumption have yet to be determined (Cravo and Camilo 2000; Mason and Choi 2005). The analysis of serum folate within the HI study sample highlighted that 40% (N=14) of participants had serum folate levels below that of the minimum clinical reference range (5 µg/l) and of these, nine were female. Serum folate levels in the ADI study sample were also low and the median level was towards the lower level of the clinical reference range (5 µg/l). There were more males in the ADI study compared to the HI study, however it was anticipated that there would be low folate levels in the alcohol-dependent patient sample as this has been established

within the published literature (Cravo and Camilo 2000; Cravo et al. 1996; Mason and Choi 2005). In the studies published which investigated the relationship between folate and alcohol dependence, Bleich et al. (2000), reported a median serum folate of 9.2 µg/l in a sample of alcohol dependent patients at admission, which was higher than the median value found in the present ADI study.

Folate is an important B-vitamin, which protects against anaemia and is crucial to the normal development of the foetus. Participants recruited to the HI study were a convenience sample from a university population. There was a gender bias in terms of recruitment as the student population of Queen Margaret University is 77% female. A folate level of below the minimum healthy clinical reference range is concerning within a sample of females of reproductive age. Interestingly, of the female participants who had folate levels below 5 µg/l, they were found to be undertaking sessional drinking. The low folate level may be the consequence of at least two factors; firstly poor diet. The poor nutritional content of the diet of some university students has been reported in the literature (Comrie et al. 2009). The second factor may be sessional drinking. The results presented here raise the possibility that young women of reproductive age may be exacerbating their poor dietary intake of folate through their alcohol drinking pattern and this question is worthy of further research.

In the ADI study, serum folate levels were shown to significantly ($p=0.007$) decrease for the total sample, as detoxification treatment progressed. Serum folate levels did increase in the participants who consumed both wine and beer, as alcohol detoxification treatment progressed. This finding of a reduction in serum folate levels with time mirrors the finding of increased plasma homocysteine level, as alcohol detoxification treatment progresses. The study by Gibson et al (2008), highlights that consumption of alcohol does significantly increase plasma homocysteine levels within responsible drinkers and agrees with published literature that as homocysteine levels increase, folate and vitamin B_{12} levels decrease (Antoniades et al. 2009; Bleich et al. 2000c). The decrease in serum folate and increase in plasma homocysteine levels found in ADI participants was expected, as it has been widely reported in the literature that there is an inverse relationship between homocysteine and folate (Antoniades et al. 2009). However this inverse relationship would be expected when the ADI participants were consuming alcohol

and not during alcohol detoxification treatment, whereby serum folate should increase in the presence of no alcohol consumption and plasma homocysteine should decrease. The ADI study questionnaire asked the alcohol dependent participants which type of alcohol they regularly consumed or was their preferred drink. There was a higher proportion of ADI participants who were dependent on only one type of alcoholic beverage (N=10). While numbers were admittedly small, the potential effect of the type of alcohol on folate levels was investigated and it was found that those who drank only spirits had higher levels of serum folate prior to entering detoxification treatment.

This finding does not agree with the published literature as, it has been shown that individuals who consume beer tend to have higher serum folate levels in comparisons to individuals who abuse alcohol using other types of alcoholic beverage (Bleich and Degner 2000; Bleich et al. 2000c). Higher serum folate levels in beer drinkers are due to the high content of folate found in this alcoholic beverage. As previously stated, ADI participants who consumed only beer did have reduced serum folate levels, as detoxification treatment progressed, however they were the only participants who were found to have a reduction in plasma homocysteine levels from day 1 to day 3. Since only two participants consumed beer, it could be suggested that the consumption of beer, even when serum folate levels are low, does still have a reducing effect on plasma homocysteine and this finding requires more investigation.

The reduction in serum folate levels, as alcohol detoxification continues suggest that participants should be supplemented with folic acid to increase the circulating levels of folate, as soon as detoxification treatment begins. The supplementation of ADI participants by the prescribing of Pabrinex and Thiamine does increase B-vitamin levels and treat symptoms of malnutrition, but does not increase the levels of folate and vitamin B_{12}. It is routine clinical practice for alcohol dependent patients to be prescribed B-vitamins, as it has been well reported in the literature that alcohol consumption depletes folate and vitamin B_{12}. The prescribing of Pabrinex and Thiamine was recorded to determine the nutritional status of the ADI study sample. The findings, reported in chapter 3, indicate that the nutritional health of the study participants was poor, as a high percentage of the sample were prescribed Pabrinex indicating that they were severely malnourished, consistent with the negative effect

of alcohol abuse. The supplementation of folic acid would reduce the negative affects associated with decreased folate levels. It would also be important to consider which type of alcohol was being consumed before treatment started, as this has shown to impact on serum folate levels. This further influences the potential for the type of alcoholic drink to affect folate, therefore having an impact on homocysteine levels.

It has been reported in the literature that serum folate levels are inversely correlated with plasma homocysteine levels in humans (Antoniades et al. 2009). Bleich et al. (2000) also reported that serum folate was inversely correlated with plasma homocysteine in a sample of alcohol dependent individuals undergoing alcohol detoxification. This relationship was suggested for the ADI study participants however, there was no significant correlation detected on day 1 ($p=0.140$, $R=-0.452$) and day 3 ($p=0.154$, $R=-0.460$). For the HI study, again the correlation coefficient was not significant ($p=0.61$, $R=-0.324$). It is important to note that the ADI participants were undergoing detoxification treatment and have been shown to be malnourished, which when compared to the HI study participants, who have not been undergoing an alcohol detoxification and have been continuing with their normal drinking and dietary intake. Therefore the ADI study sample does not represent a continuum. This area of work does require more investigation with a larger sample.

Several facts give attest to the accuracy of the serum folate results presented for both the HI and ADI studies. Levels of folate in control, pooled, serum samples stored under similar conditions to participant samples (-80 ºC storage) or subjected to multiple freezing and thawing, did not differ significantly ($p=0.858$). In addition the coefficient of variation within a batch analysis of participant's serum was confirmed to be less than 10% at each run.

4.4.2 Serum Vitamin B_{12}

Like folate, vitamin B_{12} is also an important cofactor involved in the remethylation of homocysteine. Within the HI study sample, serum levels of vitamin B_{12} were found not to change significantly during the 6 month period of the study ($p=0.139$). When

investigating the status of vitamin B_{12} within HI study individuals, with different drinking patterns, the abstainers did have higher vitamin B_{12} levels, which significantly (p=0.01) increased as the study progressed. The levels of vitamin B_{12} in the other groups; responsible drinkers, exceeding responsible guideline drinkers and sessional drinkers all had similar vitamin B_{12} levels, which were lower than the abstaining individuals, which might be anticipated, due to the depleting effect of alcohol on vitamin B_{12} (Thuesen et al. 2010). Alcohol consumption can impair the uptake or retention of vitamin B_{12} by the liver and other peripheral tissues (Cravo and Camilo 2000).

In the present work there was no significant change in serum vitamin B_{12} within each alcohol drinking pattern at the study time-points. Within the HI study sample there were three participants who had vitamin B_{12} levels below the minimum clinical cut-off value of 200 ng/l and two of these individuals consumed alcohol in a pattern which exceeded the responsible daily guidelines, suggesting a potential link to alcohol consumption.

The status of vitamin B_{12} within the ADI study sample was also similar to that of the HI study sample, whereby the serum levels for all patients were within the clinical reference range. This suggests that vitamin B_{12} is less affected by alcohol consumption in comparison to folate. The recommended daily intake (RDI) of vitamin B_{12} is very small, 2-3 µg, and can remain stored in the body for a long period of time, explaining the low RDI (Solomon 2007). Consumption of alcohol does not deplete vitamin B_{12} as readily as folate, which could explain the normal range of serum levels for this vitamin detected in both the HI and ADI studies.

It is well documented in the literature that alcohol consumption, in a heavy pattern does deplete B-vitamins (Birch et al. 2009; Brattstrom 1996). For general population adult drinkers, a recent study highlighted the fact that alcohol consumption was significantly inversely associated with low vitamin B_{12} concentration (Thuesen et al. 2010). However, surprisingly in the present study, a significant positive correlation (p=0.004, R=0.449) was detected between daily alcohol consumption in grams and vitamin B_{12} levels in drinkers (N=44) (HI and ADI study participants, excluding abstainers). A possible explanation of this apparent contradiction with previous studies is suggested by the finding that alcohol consumption in a dependent pattern

may cause falsely normal vitamin B_{12} levels in the serum, by vitamin B_{12} leaking out of hepatic cells or also by oxidative stress inactivating holotransocolabalmin (HoloTC), which is the active fraction of the vitamin B_{12} molecule (Fragasso et al. 2010). However this finding has not been established in a sample of healthy individuals, who consume alcohol in a range of non-dependent patterns. It is also important to note that, like folate, vitamin B_{12} could be affected by dietary intake and not solely affected by alcohol consumption.

Literature evidence suggests that there is an inverse relationship between plasma homocysteine and vitamin B_{12}, where a high vitamin B_{12} concentration relates to a lower homocysteine level (Antoniades et al. 2009). No significant correlation between vitamin B_{12} and plasma homocysteine was evident in the healthy individuals investigated at baseline ($p=0.596$; $R=-0.093$). However within the ADI study sample a significant inverse correlation was found on day 1 ($R=-0.594$, $p=0.042$) and improved by day 3 ($R=-0.864$, $p=0.001$). This finding suggests that vitamin B_{12} and homocysteine have a more defined relationship in alcohol dependent drinkers in comparison to 'social' drinkers. A potential explanation for this reduced correlation in the HI study sample could be that the detrimental effects of alcohol consumption are not apparent. Vitamin B_{12} is stored in the body for a long period and is less readily depleted than folate. The difference between the two drinking groups in terms of vitamin B_{12} levels could be explained by the period of alcohol consumption, whereby the longer heavy alcohol consumption has been undertaken the more reducing effect this has on stored vitamin B_{12} levels.

Investigation of an association between the diet diary vitamin B_{12} results with serum levels determined by biological assay revealed a non-significant inverse correlation ($R=-0.429$). This finding was not expected and suggests that the WinDiet software does not produce a reliable result for vitamin B_{12} analysis. This finding could be suggested as the stability study results for the vitamin B_{12} assay showed that -80℃ freezer storage and multiple thawing and refreezing did not affect the analysis of vitamin B_{12}. The vitamin B_{12} assay used, was within a hospital laboratory and the coefficient of variation was less than 10%, due to the strict variation guidelines within diagnostic laboratories. The reason for a lack of association between diet diary vitamin B_{12} analysis and serum vitamin B_{12} assay has yet to be determined, and requires further investigation. It is also important to note that the analysis of diet

diaries is dependent on the accuracy of participant recall. Recently published work by Fragasso et al. (2010), has suggested that normal serum vitamin B_{12} levels are found in alcohol dependent individuals, due to mechanisms described previously, however, this has not been determined in healthy individuals who consume alcohol in various patterns and the functionality of the vitamin B_{12} assay does require clarification in light of this recent publication.

4.4.3 The Effect of Folate and Vitamin B_{12} on Reducing Homocysteine Levels

Dietary supplementation of folic acid and vitamin B_{12}, has been shown to reduce the circulating levels of homocysteine in a number of studies and clinical trials (Al-Tahan et al. 2008; Antoniades et al. 2009; Bonaa et al. 2006; Dangour et al. 2008; Pfeiffer et al. 2008; Stea et al. 2008; The Heart Outcomes Prevention Evaluation (HOPE) 2006; Wotherspoon et al. 2008). This can have benefits in reducing homocysteine levels in patients with diagnosed cardiovascular disease (Antoniades et al. 2009). All of the studies, describing the influence of supplementation of folic acid and vitamin B_{12} on homocysteine levels, as listed in the table in appendix 4C, concluded that in humans, supplementation of combined doses of folic acid and vitamin B_{12} reduced the plasma levels of homocysteine. However, in samples where the participants had established CVD, the mortality risk was not reduced. Several authors, dispute the most effective dose to actively reduce plasma homocysteine (Antoniades et al. 2009). Current evidence suggests that a total dose of 400 µg of folic acid and 0.02-1 mg of vitamin B_{12} daily would actively reduce circulating homocysteine concentrations to within the clinical reference range of 5-15 µmol/l. Certain individuals within both the HI and ADI studies, exhibited homocysteine levels of above 15 µmol/l and it could be suggested that folic acid and vitamin B_{12} supplementation could be beneficial for these individuals.

The ability of folate and vitamin B_{12} to reduce plasma homocysteine levels is important to consider when investigating the detrimental effect of alcohol consumption on circulating levels of homocysteine. The present results have clearly shown that for some individuals who consume alcohol in a range of patterns, including dependency, folate is below the minimum clinical reference range; (40% in HI sample; 61.5% in ADI sample), which increases the risks associated with

depleted folate levels, e.g. increase in circulating plasma homocysteine. The dietary supplementation of folic acid and vitamin B_{12} (although this has been shown to be less affected by alcohol) needs to be investigated further to determine the potential of long term benefit of lowering homocysteine in a sample of known heavy drinkers (sessional drinkers and alcohol-dependent individuals). In the literature there is a lack of studies which have actively investigated the potential value of dietary supplementation of folic acid and/or vitamin $B_{12,}$ in individuals with no history of CVD, who consume alcohol in a range of patterns, including sessional drinking.

The findings from the HI study suggests that some alcohol consuming individuals who have self-reported themselves as being in good health are in fact at risk of hyperhomocysteinemia and the risks associated with this condition, the major one of which is cardiovascular disease. As folic acid and vitamin B_{12} have been shown to actively reduce homocysteine concentrations, it would be appropriate to consider further studies and trials to investigate the potential for folic acid and or vitamin B_{12} supplementation, as an intervention for reducing homocysteine levels and therefore risk of CVD, in a sample of individuals who consume alcohol in a range of patterns. If this further work, with a larger sample size was undertaken in a population where CVD was not established, a determination of homocysteine lowering on reducing mortality could be determined.

4.5 Limitations of the HI and ADI Studies

The recruitment of study participants into both studies was affected due to a number of reasons. It is important to state that all study participants did not receive payment for taking part in the study, which could have reduced recruitment potential, however no payment did encourage participants to take part for non-financial reasons. The HI study design did make particular demands on the participants, e.g. six month study timescale, three clinical appointments where multiple blood and urine samples were taken, 12 hour fasting, and recording of alcohol diary for seven days on three occasions. This likely did impact on recruitment and retention rates. The literature has suggested that participant payment or incentives can potentially increase recruitment into clinical research studies and trials (Bentley and Thacker 2004). If both studies were repeated, it could be suggested that participant payment or incentives (e.g. shopping voucher), could be offered. However it is important to note

that the inclusion of participant payment would be subject to ethical approval and the availability of funds within the study budget. The recruitment into the HI study was subject to the gender bias of QMU, which has a 77% female population, therefore as previously stated there was a higher number of females in the HI study. In terms of recruitment into the ADI study, this was limited due to the four monthly rotation of the FY1 (first year) junior doctors on the ward, which disrupted recruitment and screening of participants, as each new doctor had to be made aware of the study inclusion and exclusion criteria's. The low participant recruitment into both the HI and ADI studies, impacted on the statistical analysis and the type of analysis undertaken. The ADI study, which was designed in relation to other previous studies in alcohol detoxification clinics, did have a specific sample size, however the required sample size was not met, therefore non-parametric statistical analysis had to be used within this data set. For the HI study, there was no sample size calculation as this was a pilot study and had not been previously undertaken in the literature. The total sample size was small, and this further impacted on the type of statistical tests used. When the sample was further categorised into alcohol drinking pattern groups the sample size was further reduced and was not balanced. It is important to note that significant findings were found, but as the sample size was small, these findings can only suggest possible associations between variables and requires further investigation in a larger sample.

Problems associated with participant recruitment and retention to clinical trials and observational studies has been well documented in the published literature (Gul and Ali 2010). Recent data has highlighted that only 31% of trials (N=122) in the UK, were successful in recruiting 100% of their target sample (Free et al. 2010). This shows the difficultly in recruiting to not only major multi-centre trials but also to smaller observational studies, like the HI and ADI studies. The effect of not recruiting the target sample reduces the study power and hence important differences in clinical markers will potentially not be identified.

The HI study did not have an exact sample size, as the study was deemed pilot work, however the ADI study did have a required sample size of 31 participants. The ADI study did not recruit to target, and only recruited 58% of the required sample size. The published literature does suggest certain reasons why under-recruitment happens, however the general consensus, are the reasons for under-

recruitment is poorly understood. One possibility is that potential participants did not understand the benefit of taking part in the study/trial, not only to themselves but to the medical/scientific community as a whole (Free et al. 2010). The paper by Free et al. (2010), suggested that the study/trial documentation should be piloted to potential participant groups before study initiation, with a view to gaining their opinion. This was something that was carried out in the HI study, before recruitment began. A focus group, would allow for potential participants to voice their opinions/concern. The use of focus groups has been implemented as part of major multicentre RCTs. Another point to consider are the clinical staff, who are recruiting participants, as their experience in working with certain groups of patients, will also be integral to finding out if a study can recruit to target; this was something that was implemented in the design of the ADI study.

A number of journal papers have suggested that factors in participant's personal lives can affect their participation (Woodward et al. 2010). A conference abstract by Woodward et al. (2010) suggested that participants unwillingness to take part in research can be due to levels of trust in research staff, race (the study found significantly ($p=0.01$) more Caucasian than non-Caucasian's takes part in research) and the presence of medical insurance was a major factor in participating in US trials/studies. Furthermore the basic understanding of why the research is taking place can also hinder participant recruitment, especially if the target sample group does not understand why the research is taking place. This issue can be tackled through the use of well worded patient information sheets and the ability for potential participants to discuss their participation with their GP or an independent clinician, not involved in the study. The privacy of potential participants is another issue when taking part in research studies. Papers have reported that participants feel by taking part in research and being monitored or followed-up for a long period of time (certain studies or trials can have a follow-up phase of up to 5 years), is invading privacy (Free et al. 2010). This factor of privacy is something to especially consider when investigating alcohol consumption as both the ADI and HI study asked participants to report their drinking during a 6 month period (HI) and report their alcohol consumption prior to alcohol withdrawal treatment (ADI). The questions asked, did require participants to be specific in reporting their consumption and if there were concerned or worried about their consumption levels; this could have affected their participation within each study, hence resulting in non-participation.

To tackle the issue of under-recruitment, a collaboration of Scottish Universities and the Scottish Government designed an advertising campaign, which was broadcast throughout Scotland, with an aim to better inform the Scottish Population about RCTs and taking part in research studies (Mackenzie et al. 2010). Analysis of public opinion following the advertising campaign described by Mackenzie et al (2010), showed that there was statistically significant ($p<0.01$) better understanding of RCTs and the research study participation after the advertising campaign, however there was no statistical ($p=0.92$) difference in participants willingness to take part in research studies following the advertising campaign. This research shows that by educating the general public about research studies, their awareness of research improves, however there is more work required to improve their willingness to take part and thus prevent under-recruitment to research studies.

The individuals recruited into both studies formed a convenience sample, which was not stratified or randomly selected and were volunteers. The HI study sample can be described as being a convenience sample, due to the nature of the recruitment site. As previously described in earlier chapters, the HI study recruited from two Edinburgh universities, and the sample was entirely composed of participants who replied to the initial email and poster advertisement. Participants were not directly targeted by age, gender, drinking patterns and the advertising of the study did not ask for individuals who consumed a certain quantity or type of alcohol. Therefore the sample was composed of entirely willing and available participants within the given recruitment location. This convenience sample is unlike a clinical trial sample, where patients are recruited through multiple sites and targeted due to a specific condition or disease which makes them eligible to take part within the trial. The HI study therefore enabled data to be gathered on a sample of willing abstainers or alcohol consumers located within a major Scottish city. The healthy individuals who consumed large volumes of alcohol were unlikely to have volunteered as they would not have wanted their consumption levels scrutinised, which means the findings of the HI study are not of a typical population and are isolated within the individuals who were recruited. As previously stated there was a female bias in the HI study due to location of recruitment, meaning there was a lack of data on male alcohol consumption and subsequent comparison to biomarker results. The finding of both studies can not be generalised and they point towards interesting findings within a

representative sample of Scottish adults, who volunteered for each research study (HI and ADI). However these findings need to be investigated within a larger multi-site sample.

A major limitation of both the HI and ADI studies is memory and accuracy recall of the participants, when completing the questionnaires and diaries for both dietary and alcohol analysis. It has been well documented in the literature that memory recall can impact on accuracy of alcohol data acquired from diaries and questionnaires (Catto 2008). However the use of the analysis method to determine alcohol consumption had taken into consideration all the most relevant and required information when calculating consumption. The use of the WinDiets software, although primarily not used for alcohol consumption analysis, did highlight underestimation of alcohol consumption when used and there was no reliability or validity data available on the WinDiets software within the literature. The only true way to quantify and determinate true alcohol consumption is by watching and measuring alcohol as it is being consumed, which is highly impractical, therefore the use of an accurate and sensitive alcohol biomarker is of great importance. Another issue which was a limiting factor to take into consideration was the effect the study had on participants self-reporting of both alcohol consumption and dietary intake, the so called “Hawthorne Effect”. The Hawthorne effect can be defined as altering of behaviour or performance resulting from the participation in a research study (Campbell et al. 2005). The results of the HI study clearly showed a change in alcohol drinking patterns over a 6-month period and a reduction in alcohol consumption; however this was not statistically significant. The Hawthorne effect should be taken into consideration as a limiting factor, when investigating alcohol consumption, as this can influence the behaviour of the participants taking part in the research and effect subsequent findings.

All laboratory analysis used established methods, which had coefficient of variations below 10%. Each batch analysis for all biomarker analysis included standards and the production of a calibration curve, which ensured the functionally and accuracy of the assay. The stability work on plasma homocysteine did highlight inter-assay variation in batch analysis. This finding has a limiting factor in terms of how batch analysis should be organised, but it is important to note that this finding directly related to the maximum HPLC column usage, which suggests that the inter-assay

variation should be monitored closely in relation to the HPLC column life span. The analysis of plasma homocysteine and CDT did not use the gold-standard methods, of LC-MS and HPLC respectively. This potentially did reduce the sensitivity of the results, however neither method was available for use locally and the results produced from each method were from a suitable validated method. The laboratory analysis used for the detection of the $MTHFR_{(C677T)}$ polymorphism, folate and vitamin B_{12} were derived from the most uptodate laboratory methods.

The analysis of folate, vitamin B_{12} and CDT were conducted within hospital laboratories, which have strict guidelines on batch analysis procedures and coefficients of variation within batches, which gave added certainty to the accuracy of these biomarker results. The recent publication by Fragasso et al (2010), suggest that vitamin B_{12} analysis within an alcohol dependent patient sample produces false normal vitamin B_{12} levels, however this has not been established in healthy individual who consume large volumes of alcohol. This relatively new work, does requires more investigation and could potentially be a limiting factor in term of vitamin B_{12} analysis in a sample of heavy drinkers. The assays for the determination of homocysteine in plasma and urine could also have been limited by participant's failure to undertake the conditions required before blood sampling, whereby for plasma homocysteine analysis, participants were required to fast for 12 hours and for urine analysis, the sample must be a waking sample. All participants self-reported that they did adhere to these conditions, which again related to participant's honesty, but must be a factor to take into consideration.

CHAPTER 5: FINAL CONCLUSIONS

A comparison of the alcohol intake of participants within the HI study and the ADI study has highlighted that healthy individuals who consume alcohol in a sessional pattern can consume the same volume of alcohol as an individual who has been diagnosed as being alcohol dependent. The analysis of alcohol consumption also provided preliminary evidence, entirely consistent with published literature, that when the consumption of alcohol is being monitored, this can have a modifying effect on the amount an individual consumes.

The various methods used to analyse and calculate alcohol consumption from questionnaires and diaries has been investigated and compared in this thesis. This work has highlighted the difference between each method, namely the alcohol manufacturer (AM) method using %ABV, the WinDiets (WD) dietary software method and finally the Office of National Statistics (ONS) guidelines. The WinDiets and ONS method were both compared to the AM method, which was deemed to be more accurate as it takes into consideration the brand, the volume and %ABV alcohol content of each drink consumed. The comparison of each method to the AM method provided results which concluded that the WD and ONS miscategorised healthy individuals in relation to their drinking pattern, meaning that drinkers were being categorised as responsible drinkers when actually they were consuming alcohol in a sessional pattern. The accuracy of alcohol consumption analysis is important when relating alcohol consumption to cardiovascular disease or other health risks.

The estimation of alcohol consumption was also investigated using an alcohol biomarker, namely Carbohydrate Deficient Transferrin (CDT), in an attempt to reduce the errors associated with questionnaires and diaries, namely participant honesty and memory recall. The study findings provided new evidence for its use as an alcohol biomarker within healthy individuals. Published literature has already promoted the use of CDT as a biomarker for monitoring abstinence in alcohol abusing individuals. The evidence provided within this thesis, suggests that CDT may also have a role in estimating sessional drinking within a sample of healthy i.e. social drinkers. A linear relationship has been suggested between the number of drinking days in a 7-day period and a positive CDT test result.

The cardiovascular risk biomarker, homocysteine was investigated in plasma, in a sample of individuals who consumed alcohol in dependent and non-dependent patterns. Plasma homocysteine levels in both studies exhibited a large range, however the median was found to be within the plasma homocysteine reference range of 5-15 µmol/l. An interesting finding, which contradicts published literature, was found in the ADI study, where plasma homocysteine increased from day 1 to day 3 of detoxification. The published literature has found that plasma homocysteine levels decrease with alcohol withdrawal. However these studies have been conducted in other countries. This suggest that it is possibly a new finding within a Scottish alcohol detoxification unit and the volume of alcohol and type of alcohol is having an opposing effect on plasma levels of homocysteine during alcohol withdrawal. This would indicate increasing risk. More work is required, with a large sample size to identify the effect of alcohol consumption on plasma homocysteine levels in both healthy individuals and alcohol dependent patients, who consume alcohol in different patterns.

The presence of the $MTHFR_{(C677T)}$ polymorphism, affects homocysteine levels, increasing its concentration in circulating plasma. Participants in both the HI and ADI studies were genotyped for the $MTHFR_{(C677T)}$ polymorphism using Real-Time PCR. The results of the genotyping did not conclude that the presence of the mutant $MTHFR_{(C677T)}$ polymorphism promotes a higher circulating homocysteine level in plasma. However, the low participant numbers are relevant and further work with a much greater number of participants in both HI and ADI groups is required. However, evidence from this work does suggest that there is a linear correlation between plasma homocysteine levels and volume of alcohol consumed, in individuals who carry the mutant $MTHFR_{(C677T)}$ polymorphism. The implications of this if proven, are, that individuals who carry the $MTHFR_{(C677T)}$ polymorphism should be recommended to limit alcohol consumption to within responsible guidelines. Dietary advice for individuals found to carry the mutant $MTHFR_{(C677T)}$ polymorphism is to increase the folate content of their diet and also take a folic acid supplement, after consultation with a medical professional.

The two vitamin co-factors, folate and vitamin B_{12} which are important in the metabolism of homocysteine were also investigated in a sample of healthy individuals and alcohol dependent patients. Serum vitamin B_{12} concentrations were

found to be consistent throughout the duration of both studies and within the 200-900 ng/l clinical reference range. This suggests that alcohol consumption does not have a large impact on vitamin B_{12} status in both study samples. However folate was found to be depleted in both the HI and ADI study sample. A high percentage of both male and females in both studies had folate levels below the minimum clinical reference range of 5 µg/l in serum. This result was anticipated within the ADI study, due to previously published findings in the literature, but not in the HI study. This result suggests that alcohol consumption, out with the Governments drinking guidelines, and especially in the form of sessional drinking is having a negative effect on serum folate levels in healthy individuals. The implications may be more serious for young women of reproductive age.

The work undertaken in this thesis, has provided evidence which suggests that alcohol consumption in varying patterns can influence levels of the CVD biomarker homocysteine. More work is required to determine the true effect of alcohol drinking patterns and drink types on plasma homocysteine levels. A large-scale study, investigating alcohol drinking patterns and the type of alcohol consumed, in the general population, could provide further evidence of the impact alcohol toxicology has on plasma homocysteine, and thus CVD risk.

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APPENDIX 1

A. Sessional Drinking and "Standard Drink" Definitions

Reference for Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Alte et al. 2004)	(Alte et al. 2004)	Germany	"Binge drinking: number of days with five or more drinks"	"Pure ethanol weight proportions for beer, wine and spirits of 3.8, 8.7 and 26.1% respectively were used"
(Alvik et al. 2005)	Alvik et al. (2005)	Norway	"Binge drinking (five to seven SU po and ≥8 SU po)"	"One SU is approximately 12 to 13 g of absolute alcohol and is defined as one bottle of wine cooler, one-third litre of beer, one wineglass of wine, one sherry of fortified wine, or one liquor glass of liquor"
(Alvik et al. 2006)	(Alvik et al. 2006)	Norway	"Binge drinking was defined as at least 5 SU p.o. on at least 1 occasion"	"One SU was defined as 1 bottle of wine cooler, 1/3 L of beer, a wineglass of wine, a sherry glass of fortified wine, or a liquor glass of liquor (approximately 12-13 g of absolute alcohol)"

Reference of Paper	Reference of Definition	Country of Study	Definition	Definition of Drink
(Barnett et al. 2004)	(Wechsler et al. 1994)	U.S.A.	"Binge drinking (typically defined as five or more consecutive drinks on one occasion for men and four or more consecutive drinks for women)	None
(Braithwaite et al. 2005)	(Samet et al. 2003)	U.S.A.	"binge drinkers as those individuals who reported consuming five or more standard drinks within a calendar day"	"Standard"
(Budde et al. 2007)	(Windle 2003)	Germany	"binge drinking (i.e. more than 5 standard drinks in a row)"	"six drinks are equivalent to 72 g ethanol"
(Caetano et al. 2006)	(National Institute on Alcohol Abuse and Alcoholism 2004)	U.S.A.	"this is defined as drinking 4 or more drinks on a single occasion"	"1 ounce of ethanol (2 standard drinks)"

Reference for Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Callaci et al. 2004)	(Witt 1994)	U.S.A.	"binge alcohol drinking is a pattern of alcohol consumption resulting in highly intoxicating blood alcohol concentrations (BAC's)"	None
(Callaci et al. 2006)	(Grossberg et al. 2004)	U.S.A.	"6 or more drinks per occasion"	None
(Conigliaro et al. 2004)	(Bush et al. 1998; Gordon et al. 2001)	U.S.A.	"We examined binge drinking, alone by using the third question in the AUDIT, which asks whether the patient has had six or more drinks on one occasion"	None
(Cranford et al. 2006)	(Wechsler et al. 1994)	U.S.A.	"Standard binge measure of consuming 5 or more drinks in a row for men (4 or more drinks for women)"	"A drink was defined as a glass of wine, a bottle of beer or wine cooler, or a shot of liquor straight or in a mixed drink"

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Cunradi 2007)	(Cunradi 2007)	U.S.A.	“Binge drinker (drank 5 or more drinks on the same occasion)”	None
(Das et al. 2004)	(Das et al. 2004)	U.S.A.	“Binge drinking was defined as consuming five or more drinks on one occasion”	“A “standard drink” is 0.5 ounces of absolute ethanol”
(Dawson et al. 2004)	(Wechsler and Nelson 2001)	U.S.A.	“consumption of 4+ drinks is increasingly used as a measure of binge drinking for women”	“2.4 ounces of ethanol = 4 drinks”
(Day et al. 2002)	(Day et al. 2002)	U.S.A.	“women drank four or more drinks per occasion. This measure was dichotomized to represent binge versus nonbinge drinking”	None

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Doremus-Fitzwater and Spear 2007)	(Johnston et al. 2007)	U.S.A.	“drinking 5 or more drinks in a row within the last 2 weeks, with this episodic drinking defined as “binge drinking”	None
(Faden and Fay 2004)	(Johnston et al. 2002)	U.S.A.	“heavy episodic drinking (frequently called binge drinking and defined as consuming five or more drinks on one occasion in the past two weeks”	“a “drink” means “more than just a few sips”
(Faden 2006)	(Johnston et al. 2003)	U.S.A.	“binge consumption (5+ on an occasion)”	None
(Flynn et al. 2003)	(Flynn et al. 2003)	U.S.A.	“binge episodes (“How often have you had five or more drinks on one occasion?”)”	“a drink was defined for the participants as one 12-ounce beer, one 4-ounce glass of wine, and one 1-ounce shot liquor”

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Gauthier et al. 2005)	(Gauthier et al. 2005)	U.S.A.	"consuming more than four drinks in a single day, the frequency of binge drinking"	None
(Gmel et al. 2006)	(Gmel et al. 2006)	Switzerland	"Are they drinkers who usually drink heavily or heavy episodic drinkers (HED, also called binge drinkers)"	"frequency of having had 5+ drinks (men) or 4+ drinks (women) in the past month"
(Goldman 2006)	(National Institute on Alcohol Abuse and Alcoholism 2004)	U.S.A.	"consumed 5 drinks on an occasion could vary widely depending on the values of these variable. This concern was reflected in the modified definition of binge drinking issued by the National Association on Alcohol Abuse and Alcoholism"	None

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
Haley et al. (2006)	(Haley et al. 2006)	U.S.A.	“binge drinking behaviour (i.e. 4 or more drinks per drinking day)”	“drinks were converted into standard drink units 0.5 oz of ethanol”
(Handmaker et al. 2006)	(Floyd and Sidhu 2007)	U.S.A.	“bingeing (i.e. 5 or more drinks per occasion”	“standard units of 0.5 ounces of ethanol”
(Hartzler and Fromme 2003)	(Hartzler and Fromme 2003)	U.S.A.	“binge drinking (at least five consecutive drinks for men and at least four consecutive drinks for women)”	“drinking quantity is the standard drinks consumed per drinking day”
(Kable and Coles 2004)	(Kable and Coles 2004)	U.S.A.	“maternal binge drinking or self-reported drinking five or more drinks per occasion during pregnancy”	None
(Kelly et al. 2004)	(Kelly et al. 2004)	U.S.A.	“binge drinking (drinking six or more drinks at a time)	None
(Kim et al. 2007)	(Kim et al. 2007)	U.S.A.	“Binge drinking defined as consuming 5 or more drinks in a single day”	“alcohol containing”

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(King and Epstein 2005)	(King and Epstein 2005)	U.S.A.	“Binge is defined as five or more drinks/occasion for males and four or more drinks/occasions for females”	None
(Kishikawa et al. 2005)	(Malyutina et al. 2002)	Japan	“”Binge drinking” is defined as the consumption of 160 g/day or more or pure ethanol usually lasting for a week and over”	The term “drink” not used in the definition of binge
(Kokotailo et al. 2004)	(Kokotailo et al. 2004)	U.S.A.	“4 or more occasions when 5 or more drinks were consumed in 1 sitting (binge drinking)”	“one standard drink was 12 ounces of beer, 5 ounces of wine, or 1.5 ounces of hard liquor”

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Kristjanson et al. 2007)	(Kristjanson et al. 2007)	Russia	“they were asked how many times they had consumed 5 or more drinks (of any type or combination of types of alcoholic beverages) on an occasion during the past 30 days”	“One glass of vodka was assumed to contain 50 ml, 1 glass of dry wine 150 ml, 1 glass of fortified wine 90 ml, and 1 can of beer 330 ml”
(Kushner et al. 2005)	(Kushner et al. 2005)	U.S.A.	“drinking binge (i.e. four standard drinks for women and five for men in a single drinking episode”	“Standard”
(Lange et al. 2004)	(Murray et al. 2002)	The Netherlands	“rapid intake of large quantities of alcohol in a short period of time (binge drinking)”	“66 g of alcohol in the Bacardi Breezer group and 69 g of alcohol in the red wine group”
(Louise Floyd et al. 2006)	(Louise Floyd et al. 2006)	U.S.A.	“binge drinking (5 or more drinks per drinking occasion)”	None

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Lukas et al. 2005)	(Lukas et al. 2005)	U.S.A..	“binge drinking that often exceeds 10 to 12 beers per episode”	“10-12 beers”
(May et al. 2006)	(May et al. 2006)	Italy	“heavy episodic drinking (e.g. “binge drinking”)”	“Standard”
(Miller and Spear 2006)	(Johnston et al. 2005)	U.S.A.	“Binge drinkers (defined in this survey as the consumption of 5 or more drinks in a single episode”	None
(Molina et al. 2007)	(Molina et al. 2007)	U.S.A.	“5 or more drinks consumed per drinking occasion (sometimes referred to “binge drinking”)	None
(Monti et al. 2004)	(Wechsler et al. 1994)	U.S.A.	“Binges are sometimes defined as drinking five or more alcoholic drinks on an occasion”	None

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Parks and Fals-Stewart 2004)	(Wechsler et al. 1994)	U.S.A.	“binge drinking (four or more drinks/occasion)”	None
(Pelc et al. 2002)	(Pelc et al. 2002)	Belgium, Austria, UK, Portugal and Switzerland	“binge (an episode of drinking limited to 2 to 4 days between two consecutive visits)”	None
(Reinert and Allen 2007)	(Reinert and Allen 2007)	U.S.A.	“Binge drinking ≥ 8 units alcohol/d”	Drink defined in units
(Reynaud et al. 2005)	(National Household Survey on Drug Abuse (NHSDA) 2002)	U.S.A.	“”binge drinking” (i.e., consuming five or more drinks at a single setting for men and four or more drinks at a single setting for women)”	None

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Riley et al. 2003)	(Riley et al. 2003)	U.S.A.	“binge drinking (5 or more drinks per occasion)”	1.0 oz of absolute alcohol (AA) per day
(Ristuccia et al. 2007)	(Johnston et al. 2003)	U.S.A.	“binge ethanol use (the consumption of 5 or more drinks in a row)”	None
(Russell et al. 2004)	Russell et al. (2004)	U.S.A.	“heavy drinking rates (days of 5+ drinks/drinking days x 100)	“standard drinks are defined as containing approximately 0.6 ounces or 14 g of ethanol”
(Selin 2003)	(Selin 2003)	Sweden	“frequency of binge drinking (drinking at least one bottle of wine or a corresponding quantity of other alcoholic beverages on one occasion)”	“One bottle of wine or a corresponding quantity of other alcoholic beverages on one occasion”
(Silvers et al. 2003)	(National Household Survey on Drug Abuse (NHSDA) 2001)	U.S.A.	“binge drinking (consumption of five or more drinks on one occasion)”	None

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Slawecki and Ehlers 2005)	(Substance Abuse and Mental Health Services Administration 2004)	U.S.A.	“binge drinkers; that is they consumed more than five drinks in a single drinking episode within the past 30 days.”	None
(Stein et al. 2005)	(National Institute on Alcohol Abuse and Alcoholism 1995)	U.S.A.	“Hazardous drinking was scored 1 if the participant reported 1 or more binge drinking episodes or if the participant exceeded an average of 14 drinks (7 drinks for women) per week”	None
(Stewart et al. 2005)	(Stewart et al. 2005)	U.S.A.	“binge drinking (drinking five or more alcoholic beverages per occasion for boys and four or more for girls)”	“one drink equals one 12 oz bottle/can of beer, or one small 4 oz glass of wine, or one 1 oz shot of hard liquor, either straight or with a mixer”

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Tokunaga et al. 2006)	(National Household Survey on Drug Abuse (NHSDA) 2002)	U.S.A.	“binge alcohol drinking (5 or more drinks on 1 occasion)”	None
(Townshend and Duka 2005)	(Townshend and Duka 2005)	U.K.	“binge drinking behaviour is the consumption of large amounts of alcohol within a limited time period followed by a period of abstinence”	“Unit”
(Valencia-Martin et al. 2007)	(Strategy Unit Alcohol Harm Reduction Project 2003)	Spain	“Binge drinking was defined as the intake of 8 or more standard units of alcohol in men, and 6 or more in women, during any drinking session (1 evening or 1 night)”	“standard unit has been estimated as 10 g of pure alcohol; thus, binge drinking amounts to an intake of ≥80 g of alcohol in men and ≥60 g of alcohol in women”

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(van den Wildenberg et al. 2006)	(van den Wildenberg et al. 2006)	The Netherlands	“binges (defined as 5 drinks or more in a drinking episode)”	Standard
(van den Wildenberg et al. 2007)	(van den Wildenberg et al. 2007)	The Netherlands	“binges (6 drinks or more on 1 occasion)”	“A standard drink contains 10 g alcohol/U”
(Varlinskaya and Spear 2004)	(Varlinskaya and Spear 2004)	U.S.A.	“binge drinking patterns (i.e. five drinks or more in a row)”	None
(Wechsler and Nelson 2006)	(Johnston et al. 2005)	U.S.A.	“binge drinking as a pattern of alcohol consumption characterized by 5 or more drinks in a row for males (and 4 or more drinks in a row for females” during the past 2 weeks”	None

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(White et al. 2005)	(Wechsler and Nelson 2001)	U.S.A.	"Harvard School of Public Health determined that four drinks per session for a woman and five drinks per session for a man serve as thresholds for risky, or binge, drinking"	"standard drink as a 12 oz beer, a 4 oz glass of wine, or 1.25 oz of liquor in a shot or mixed drink"
(White et al. 2006)	(Wechsler et al. 1994)	U.S.A.	"binge drinking implies consuming large amounts of alcohol in a relatively short period of time. In studies of college drinking, the term refers to a dichotomous variable defined by meeting or exceeding a threshold, such as 4 or more drinks (4+) for females and 5 or more drinks (5+) for males"	"a drink was defined as a 12 oz of 5% beer, 5 oz of 12% wine, or 1.5 oz of 40% liquor in a shot or mixed drink"

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Wilhelmsen et al. 2005)	(Wilhelmsen et al. 2005)	U.S.A.	"binge drinking (consuming five or more drinks on the same occasion)"	"beer (number of 12 oz. cans), wine (number of 6 oz. glasses), or liquor (number or shots)"
(Windle 2004)	(Windle 2004)	U.S.A.	"Binge drinking was measured with a survey question requesting how many times the adolescent has consumed six or more drinks in a single sitting over the past 6 months"	"Questions were asked separately for beer, wine, and hard liquor and were then summed to form single measure of binge drinking"
(Woerle et al. 2007)	(Woerle et al. 2007)	U.S.A.	"Binge drinkers were defined as those who reported having ≥ 5 drinks on at least 1 occasion"	None

Reference of Paper	Reference for Definition	Country of Study	Definition	Definition of Drink
(Wood et al. 2007)	(Wechsler et al. 1994)	U.S.A.	""binge" drinking for that day (operationalized for men as consuming 5 or more drinks and for women as consuming 4 or more drinks in a single sitting)"	Standard
(Yuan et al. 2004)	(Yuan et al. 2004)	Denmark	"Binge drinking was defined as an intake of eight or more drinks on one occasion"	"standard quantities of alcoholic beverages in Denmark (12 g per unit, equal to 0.42 oz of absolute alcohol)l"

B. Table of 2008-2009 Studies Investigating the Relationship between Alcohol Consumption and Homocysteine

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Devika et al. 2008) India	Investigate serum homocysteine in individuals undergoing alcohol withdrawal.	N=50. Patients with existing liver disease not included. Patients abused alcohol for 8-10 years. Undergoing 8 week detoxification program. Group 1 completed 8 week detoxification (N=39). Group 2 did not complete detoxification (N=11).	Patient's diet supplemented with folic acid, vitamin B_6 and B_{12}. Blood samples taken an admission and at end of 8 week detoxification. Homocysteine analysed by ELISA. Other markers analysed by an auto-analyser.	Homocysteine levels significantly decreased ($p<0.002$) to normal levels; GGT ($p<0.02$) and ALT ($p<0.03$) levels also decreased at end of 8 week detoxification. Homocysteine at admission 26 ±13.36 µmol/l. Homocysteine after detoxification 14.31 ± 5.7µmol/l.	Study suggests that after 8 week abstinence program and vitamin B supplementation, homocysteine can be lowered in an alcoholic patient group.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Gibson et al. 2008) Northern Ireland	Investigate the effect of a 2 week intervention of red wine and vodka on levels of total homocysteine.	Participants consumed no alcohol for 1st two weeks. Healthy male volunteers n=85. Aged between 21-70 years. Participants did not drink more than 21 units/week, have abnormal liver profiles and did not take vitamin supplements	For two weeks, participants consumed either 240 ml of 12% red wine or 80 ml of 37.5% Smirnoff vodka (24 g or 3 units/day). Participants finished study with a two week alcohol free period. Vodka drinkers were told not to use fruit juices as mixers. Blood sampling taken at baseline and at end of 2 week alcohol drinking period. Plasma homocysteine analysed using HPLC-FD. Folate and Vitamin B_{12} analysed using radioimmunoassay. MTHFR polymorphism identified using PCR and HinFl digest.	Homocysteine increased after 2 week red wine intervention by 5% ($p=0.03$), but no significant increases in homocysteine after vodka intervention ($p=0.09$). Folate and B_{12} did not differ significantly between interventions.	After 2-week intervention folate and vitamin B_{12} decreased, but homocysteine increased. There was no difference in the levels of homocysteine, folate or B_{12} between vodka and red wine drinkers.

C. Table of 2008-2009 Studies Investigating the Relationship between Folate, Vitamin B_{12} and Homocysteine

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Stea et al. 2008) Norway	Investigate the effects of homocysteine, folate and vitamin B_{12} by increasing vegetable content of diet.	N=541 males aged 18-26 years. Intervention group = 416 males. Control group of 105 males. Intervention length 5 months.	Participants were asked to keep food diaries. Homocysteine and riboflavin were analysed by HPLC. Vitamin B_{12} and folate analysed by immunoenzymatic assay.	Intervention reduced homocysteine levels by 10% (p=0.002). Decrease of homocysteine was positively related to change in cysteine and inversely related to change in concentration of folate (p=0.021).	Study showed intervention which increased vegetables, fruit and bread intake, had positive effect on reduction of homocysteine levels. Also change in cysteine folate and flavo-coenzymes are a predictor of change in homocysteine levels.
(Dangour et al. 2008) UK	Investigate association of folate, vitamin B_{12} and homocysteine in relation to death from all causes and CVD in older individuals.	N=853 males and females aged >75 years	Homocysteine analysed by fluorescence polarization immunoassay. Vitamin B_{12} and folate analysed by Becton Dickinson Simultrac kit.	Individuals with plasma homocysteine levels in top one third had 2 times higher risk of all cause mortality in comparison with the individuals in the bottom one third. No association in folate, vitamin B_{12} and mortality.	Results extend previously published findings of homocysteine and mortality rate and the absence of any link between folate and vitamin B_{12} and mortality.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Wotherspoon et al. 2008) UK	Investigate the impact of oral folic acid supplementation upon plasma homocysteine levels, endothelial function and oxidative stress in patients with type 2 diabetes and microalbuminuria.	N=16 patients. 2 months of oral supplementation. Patients randomised to either group 1 receiving 5 mg of folic acid or group 2 received placebo.	Baseline sampling and sampling after intervention. HbAic measured using HPLC. Urine creatinine by Jaffe reaction. Homocysteine by LC.	Plasma homocysteine fell by 25% in the folic acid group but there was no difference in endothelial function or markers of oxidant stress in the treatment group.	Study concluded that oral folic acid supplementation successfully lowered plasma homocysteine levels; however this was not associated with improvements in endothelial function or markers of oxidative stress.
(Pfeiffer et al. 2008) USA	Investigate the total homocysteine data in the pre-fortification NHANES III and the in 3 post-fortification survey period in a national sample of the US population.	Data adjusted from original survey.	Homocysteine in plasma analysed at the DCD nutrition laboratory using a commercial florescence polarization immunoassay reagent set.	Mean plasma total homocysteine decreased by 8, 9 and 10% for adolescent, adult and older men by 6, 3 and 13%. Plasma total homocysteine concentrations (>13 µmol/l) for older men and women decreased from pre-fortification (14% and 5% respectively).	After adjusting for method changes, pre-fortification to post-fortification decreases in circulating total homocysteine concentrations of approximately 10%. Similar effects seen in smaller intervention trials.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Al-Tahan et al. 2008) Spain	To investigate and assess the status of folate, Cobalamin and homocysteine in health Spanish adolescents	N=165 (84 females; 81 males; range 13-18.5 years).	Serum Cobalamin and folate determined using Abboy Imx auto analyser. Total homocysteine measured using fully automated fluorescence polarization immunoassay. MTHFR detected using PCR and allele specific restriction digestion of the amplified products with the restriction enzyme Hinfl	Males had markedly higher homocysteine (males 8.92 5.51-22.94) µmol/l; females 7.91 (5.09-13.86) µmol/l); whereas females showed higher serum cobalamin concentration (males 540.00 (268.00-946.47) pmol/l; females 594.82 (280.63-1,559.64) pmol/l). Adolescents with the homozygous carrier of MTHFR displayed significantly higher homocysteine and lower serum folate; normal 5.73 (3.09-10.73) ng/ml serum folate, 7.57 (4.94-12.94) µmol/l homocysteine; homozygous 4.10 (2.75-7.88) ng/ml serum folate, 10.83 (7.00-22.82) µmol/l homocysteine.	The study provides data on levels of folate, coablaim and homocysteine in Spanish adolescents.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Tanaka et al. 2009) USA	To investigate genetic factors that affect circulating vitamin B_6, vitamin B_{12}, folate and homocysteine, a genome-wide association analysis.	InCHIANTI N=1175, SardiNIA N=1115, BLSA N=640. Meta analysis	Not stated.	The association of MTHFR, a gene consistently associated with homocysteine was confirmed in this meta-analysis. The ALPL gene likely includes the catabolism of vitamin B_6 while FUT2 interferes with absorption of vitamin B_{12}.	The study findings highlight the mechanisms that affect vitamin B_6, vitamin B_{12} and homocysteine serum levels.
(Mitchell et al. 2009) USA	Evaluate a method based on stable isotope dilution liquid chromatography-multiple reaction monitoring/mass spectrometry (LC-MRM/MS) for the detection of folate and homocysteine phenotype	No sample number of participants given. Blood samples taken from premenopausal women.	LC-MRM/MS used to quantify RBS folate derivatives 5-methyltetrahydrofolate (5-CH_3-THF), tetrahydrofolate (THF) and 5,10-methenyltetrahydrofolate (5,10-methenylTHF).	RBS 5-CH_3-THF had a reciprocal relationship with total homocysteine (p=0.0003), whereas RBC THF and RBS, 5,10-MTHF had direct relationships (p=0.01, 0.04 respectively). In combination these three variables accounted for 42% of the variation in total homocysteine levels.	Study concluded that a robust method for the detection of RBC 5-CH_3-THF will improve the folate/homocysteine phenotyping in patient management. This method will provide less error and provide more powerful statistical evidence.

D. ***Table of 2008-2009 Studies Investigating the Relationship between the MTHFR$_{(C677T)}$ polymorphism and Homocysteine***

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Nagele et al. 2008) Austria	To investigate if individuals with MTHFR$_{(C677T)}$ polymorphism develop higher plasma homocysteine concentrations after nitrous oxide anaesthesia compared with wild type individuals.	N=140 healthy participants. Aged over 18 years. All patients were given nitrous oxide (N_2O) as a general anaesthetic for a surgical procedure.	Homocysteine analysed by microparticle immunoassay (Abbott). Folate and Vitamin B_{12} were analysed by radioassay. MTHFR polymorphism analysis by PCR and restriction enzymes.	Patients with homozygous MTHFR (C677T or A1298C) (N=25) developed plasma concentrations of 14.9 (10-26.4) µm compared to the wild-type and heterozygous (N=115) of 9.3 (7.5-15.5) µm. After dose of NO homocysteine concentration tripled in individuals who were homozygous for MTHFR.	Study found that patients with the MTHFR polymorphism, either C677T or A1298A were at risk of developing higher plasma homocysteine concentration after undergoing nitrous oxide anaesthesia. . Individuals who possessed the MTHFR polymorphism were the only patients who's homocysteine concentration reached abnormal levels of >15 µm.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Alessio et al. 2008) Brazil	Investigate the polymorphism in the gene cystathionine ß-synthase (CBS) and the prevalence in Brazilian children in relation to homocysteine, vitamin B_{12} and folate, who have been previously genotyped for MTHFR, methionine synthase recductase (MTRR)	N=220 healthy children. 129 females and 91 males. Age range 1-8 years (mean = 4.8 years).	Homocysteine analysed by HPLC-FD. Folate and vitamin B_{12} analysed by elctrochemiluminescence immunoassay. CBS gene polymorphism detected by PCR and restriction enzymes.	19.5% of population were heterozygote for the 844ins68 polymorphism. In children who had the insertion, also carried the CBS T833C mutation. Mutations 677TT, 1298AA and 68WW are associated with an increase in homocysteine concentration. Folate and Vitamin B_{12} were not significantly different in these groups.	Study suggests a genetic influence in the concentration of homocysteine that is not dependent on folate or vitamin B_{12}.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Yang et al. 2008) USA	Assess the prevalence of genetic polymorphisms which have a relation to serum folate and homocysteine levels.	DNA samples from 6793 participants who participated in the 3rd National Health and Nutrition Examination Survey (NHANES III). The samples were genotyped for the following: MTHFR C677T, A1298C, MTRR 66AG, cys 844ins68.	The influences of the above genotypes on folate and homocysteine levels were analysed by age, sex, folate intake and 3 specific race-ethnicity groups. Folate and vitamin B_{12} analysed using a radioprotein binding assay. Homocysteine determined using HPLC.	All race-ethnicity groups, serum folate and homocysteine concentration were significantly related to the $MTHFR_{(C677T)}$ genotypes. Participants who posses the $MTHFR_{(C677T)}$ polymorphism had a 22.1% lower serum folate and a 25.7% higher homocysteine than the individuals with the CC genotype. Moderate daily folic acid intake (mean 150µg/d; 95%CI:138,162) significantly reduced the difference in mean homocysteine levels between the MTHFR CC and TT genotypes.	Study concludes that the $MTHFR_{(C677T)}$ polymorphism was associated with significant differences in serum folate and homocysteine concentrations in the US population before folic acid fortification. The effect of MTHFR 677CT on homocysteine concentrations was reduced by moderate daily folic acid intake.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Naess et al. 2008) Norway	Investigated the homocysteine levels and the MTHFR polymorphisms before the event of first venous thrombosis in the general population.	Case-cohort designed study. N=66,140 from the Norwegian Health Study of NorsTrondelag (HUNT2). Samples were analysed at 33 month before the VT event.	Serum homocysteine and MTHFR genotyping. Serum homocysteine was analysed using automated HPLC with reverse phase separation and fluorescence detection. MTHFR polymorphism was detected using RFLP, PCR and restriction enzyme digestion enzyme HinFl. Folate was determined within a hospital laboratory using the Architect Folic Acid assay.	There was no graded association with VT over quintiles of homocysteine. In men the OR was 2.17 (95% CI 1.20-3.91) for levels above versus below the 95th percentile, but no association was found in women (OR 1.00). The MTHFR C677T genotype was not related to risk for VT.	In conclusion elevated homocysteine levels in the general population did predict subsequent first VT in men but no in women.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Grassi et al. 2008) Italy	Investigate the novel genetic factors on plasma levels of total homocysteine and fibrinogen	N=302, comprised of a complex segmental analysis of a highly selected group of 44 families. Each family included at least one member with fatal premature (<50yrs) IHD.	HPLC measured using HPLC. FIB measured suing prothrombin time derived method using an automated centrifugal coagulometer. MTHFR genotyping using PCR and restriction digestion with HINfI.	Total homocysteine and FIB levels were influenced by two major genes. A significant total homocysteine latent class-MI association (OR=3.24; 95% CI, 1.37 to 7.68) and non-significant total homocysteine level MI-association (OR=1.65 per 1-log 10 µmol/l, 95% CI, 0.56 to 4.81) were estimated suggesting a direct influence of the homocysteine major gene as a supressor plasma total homocysteine levels.	Concluded evidence for a major latent gene effect influencing variation in total homocysteine plasma levels, which is independent on C677T MTHFR polymorphism and significantly affecting the risk of MI.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Barbosa et al. 2008) Brzail	Investigate the association between MTHFR (C677T and A1298C), MTR A2756G and MTRR A66G gene polymorphism and total homocysteine, methymalonic acid, and SAM/SAH levels and the potential interactions with folate and cobalamin.	N=275 females, sampled at labour and gave birth to health babies.	All markers measured in serum. Polymorphism determined using PCR and RFLP. Folate determined using ion capture method and by chemiluminscnet method. Cobalamin concentrations were measured using the Immuliet kite. Total homocysteine was determined using the GCMS.	Serum folate, MTHFR C677T allele and MTR 2756AA genotypes were the predictors of total homocysteine levels in pregnant women. Low levels of serum folate were associated with elevated total homocysteine in pregnant women, independently of the gene polymorphism. In pregnant women carrying MTHFR 677T allele, or MTHFR 1298AA or MTRR 66AA genotypes, lower cobalamin levels were associated with higher levels of total homocysteine.	Serum folate and MTHFR C677T and MTR A25576G gene polymorphisms were the determinants of total homocysteine levels. The interactions between low levels of serum cobalamin and MTHFR or MTRR A66G gene polymorphism was associated with increased total homocysteine.

E. Table of studies published in 2008-2009 investigating the relationship between homocysteine and cardiovascular disease.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Keles et al. 2009) Turkey	Investigate plasma homocysteine levels in patients with early formation of angiographically visible collaterals after acute myocardial infarction.	N=60 participants (47 males and 13 females). Age range 32-77 years. All patients had ST-segment elevation myocardial infarction and underwent coronary angiography.	Total homocysteine levels determined using HPLC with Fluorescence detection.	No statistical difference between participants in relation to gender, age, lipid profile, prevalence of hypertension, diabetes mellitus and smoking. Plasma homocysteine concentration in patients with poor collateral formation was 18.2±8.6 µmol/l. Patients with good collateral formation had homocysteine level of 12.7±2.4 µmol/l. 24 participants had a plasma concentration that was above normal range of >15 µmol/l, of which 20 had poor collateral formation. Homocysteine levels were the only independent variable that affected the development of collaterals (p=0.003).	Inverse relationship between plasma homocysteine levels and the formation of collateral development after MI.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Vaya et al. 2008) Spain	Investigate homocysteine levels in patients with confirmed DVT.	DVT group (N=155). Control group (N=153). Healthy subjects with no previous history of thrombotic events. All Caucasians. Sampling took place 6-months after thrombotic event.	Homocysteine measured by fluorescence polarization immunoassay (FPIA).	No significant different between control and DVT group in relation to homocysteine levels (p= 0.233). DVT group homocysteine = 10.1±3.7 µM. Control homocysteine 10.2±2.8 µM.	Study suggests hyperhomocysteinema is not an independent risk factor for DVT. Also the Mediterranean diet can impact on homocysteine levels, due to a diet large in fish, fruit and vegetables.
(Cui et al. 2008) Japan	Investigate the association of serum homocysteine and cardiovascular disease in an Asian population.	N=38158 (13382 males, 24776 females). Participants had no cardiovascular disease histories.	Homocysteine analysed using HPLC. Serum total and high density lipoprotein cholesterol was measured using enzymatic methods.	At 10 year follow-up there were 444 cardiovascular related deaths. Participants within the highest total homocysteine quartile (>15.3 µmol/l) had higher mortality, in comparison with the lower quartile (<10.5 µmol/l)	High serum total homocysteine level were associated with increased mortality from ischemic stoke, coronary heart disease and total cardiovascular disease among a Japanese population.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Shammas et al. 2009) USA	Evaluation of the biomarkers of combined non-fatal myocardial infraction and end point death, 2 years after coronary artery bypass grafting.	N=121. Patients included who were undergoing bypass surgery for coronary artery disease. Two groups: no symptoms after surgery; asymptomatic after surgery including non-fatal MI.	No laboratory methodology stated. The patients had blood sampling taken 1 week before surgery and at 2 year follow up.	90 patients remaining of original cohort after 2 years. Univariate analysis showed significantly higher homocysteine level in patients who met the combined end point versus those that did not (11.0 ng/mol vs 9.7 ng/mol) $p<0.05$. Logistic regression analysis modelling indicated homocysteine is an independent predictor of primary combined end point ($p=0.016$).	Study suggests homocysteine is an independent predictor of the combined end point of cardiovascular death, non-fatal MI and vein graft disease 2 years after bypass surgery.
(Albert et al. 2008) USA	Investigate intervention of vitamins B_6, B_{12} and folic acid supplementation in high risk women with and without CVD.	N=5442 females. Aged 42 years and over with history of CVD or 3 or more coronary risk factors. Daily dose of 2.5 mg folic acid, 50 mg B_6 and 1 mg vitamin B_{12}.	RCT of combination pill or placebo. Homocysteine analysed by enzymatic assay and folate analysed by immunoassay.	Intervention group plasma homocysteine was decreased by 18.5% ($p<.001$), by a difference of 2.27 µmol/l in comparison with placebo group.	After the 7.3 year intervention, the combination pill did not decrease the risk of CVD events in high risk females, despite a significant decrease in homocysteine.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Freitas et al. 2008) Portugal	Investigate prevalence of MTHFR polymorphism in population of individuals with CVD.	N=510 healthy individuals with no history of CVD as control group. N=298 individuals in CVD group. CVD confirmed using angiography.	MTHFR polymorphisms identified using PCR and restriction enzymes. Homocysteine in plasma analysed using HPLC.	Homocysteine was independently correlated with CVD. Homocysteine was significantly higher in individuals who had MTHFR polymorphism C677T and 1298AA. The presence of both polymorphic genotypes suggests an increase in the risk of developing CVD. In study population there was a higher presence of the 1298AA genotype and CVD.	Study suggests that the presence of the 1298AA and combination of C677T and 1298AA polymorphism within the MTHFR enzyme, have a 1.6 fold increased risk of developing CVD in Madeira.
(Onat et al. 2008) Turkey	Investigate serum homocysteine levels in association with coronary artery disease and metabolic disease in a Turkish population.	N=338 men, N=342 females, with median age 55 years.	Homocysteine was measured using nephelometry. Vitamin B_{12} and folate analysed using chemiluminscent immunometric methods.	Serum homocysteine concentration was 12.7 ± 1.5 µmol/l in men and 9.6 ± 1.4 µmol/l in women. Homocysteine levels in men were independently associated with CHD. Folate levels in men were shown to have a significant inverse association with CHD.	Study suggests in Turkish men, high folate and high serum homocysteine levels are independently associated with CHD. In Turkish women, vitamin B_{12} concentrations are significantly associated with metabolic syndrome.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Foussas et al. 2008) Greece	Investigate homocysteine levels on the long-term cardiovascular mortality in patients with ST-segment elevation myocardial infarction (STEMI) or non-ST-segment elevation acute coronary syndromes (NSTE-ACS).	N=458 STEMI group and N=476 NSTE-ACS group. Patients who presented within the first 12 to 24 hours of index pain were studied.	Homocysteine and cardiac troponin I (cTnI) were measured using enzyme based immunoassay. C-reactive protein was measured using nephelometric method.	No difference in the risk of 30 day cardiovascular death among the tertiles of total homocysteine in patients with STEMI (7.2%, 8.5%, and 12.4% for the first second and third tertiles respectively; p_{trend}=0.3. Patients in the upper total homocysteine tertile were at a significantly higher unadjusted risk of late (from 31 days through 5 years) cardiovascular death than those in the other two tertiles in STEMI (23.4%, 27.9% and 41.8% for the first, second and third tertiles respectively; p_{trend}<0.001).	Data from the study indicates that total homocysteine levels which were determined on admission do not serve as an independent predictor of long term cardiovascular mortality in patients who have STEMI or NSTE-ACS.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Sosin et al. 2008) UK	Study hypothesised that ethnic differences in homocysteine are present in patients with systolic heart failure.	N=112, UK multiethnic individuals with systolic heart failure. Control matched subjects, N=131.	Homocysteine, folate, and vitamin B_{12} measured using the ADVIA Centaur automated immunochemistry system.	Plasma homocysteine levels were significantly higher in heart failure patients compared to controls ($p<0.001$), which was consistent across all ethnic groups. No significant different in levels of folate and vitamin B_{12} in patients and controls. A stepwise linear regression model showed homocysteine levels in patients and controls were independently associated with age ($p<0.001$), vitamin B_{12} ($p<0.001$); folate ($p<0.001$) and health control status ($p<0.001$), but not with gender, ethnicity, diabetes status, smoking status or BNP levels.	Study suggests that there is no evidence of ethnic differences in homocysteine levels between the following ethnic groups: White European, south Asian and African Caribbean patients who have systolic heart failure. The study showed that there no difference between levels of folate and vitamin B_{12} in patients and control groups which indicates that the level of homocysteine could be driven by dietary factors.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Ebbing et al. 2008) Norway	Investigate the dosing of folic acid, vitamins B_{12} and B_6 on patients with coronary artery disease and aortic valve stenosis.	Randomised double-blind controlled trial. N=3096 adult participants who were undergoing coronary angiography. 20.5% of study population female, mean age 61.7% were randomised.	Participants randomised into 4 groups: group 1 received folic acid 0.8 mg, vitamin B_{12} 0.4 mg, vitamin B_6 40 mg (N=772). Group 2 received folic acid plus vitamin B_{12} (N=772). Group 3 received vitamin B_6 (N=772). Placebo (N=780). All laboratory markers analysed within hospital laboratories. No techniques stated.	Mean plasma total homocysteine concentrations were reduced by 30% after 1 year of treatment in the groups receiving folic acid and vitamin B_{12}. The trial was terminated early because of concern among participants due to preliminary results from a contemporaneous Norwegian trial suggesting adverse reactions from the intervention.	The trial did not find any effective results for the treatment of folic acid/vitamin B_{12} or vitamin B_6 on total mortality or cardiovascular events. The findings gathered before termination did not suggest the use of vitamin B as a secondary prevention of patients with coronary artery disease.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Collings et al. 2008) Finland	Investigate whether common risk variables interact with the Connexin (Cx37) polymorphism causing possible interactions with early markers of atherosclerosis	N=1440 population from the cardiovascular risk in young Finn's study. Young adult sample.	Homocysteine was determined using mircoparticle enzyme immunoassay kit.	Homocysteine in subjects with the TT genotype were found to be associated with higher flow mediated dilation (FMD) values (p for interaction 0.038).	Study concluded the effect of smoking and homocysteine levels on arterial endothelial functions and elasticity were modified by the allelic variation of the Cx37 gene.
(Rassoul et al. 2008) Germany	Aim to investigate the relationship between the severity of CAD and the MTHFR polymorphism.	N=113 male patients undergoing coronary artery bypass surgery. Mean age 63±11 years.	Serum homocysteine was measured by HPLC-FD. MTHFR identified using PCR. Serum folate/ vitamin B_{12} by chemiluninscene.	Patients with CAD showed a significantly higher serum concentration of homocysteine than control subject ($p<0.01$). The serum homocysteine level was significantly higher in patients' with increased scores than in patients with mild CAD, with and without the MTHFR polymorphism.	Study has shown that hyperhomocystemia was significantly related to the severity of CAD independently of MTHFR polymorphism.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Yeter et al. 2008) Turkey	Investigate the relationship between plasma homocysteine levels and ST segment resolution (STR) after fibrinolytic therapy.	N=49 patients, all administered fibrinolytic therapy for ST-segment elevation myocardial infarction (STEMI). Group 1 N=15, group 2 N=34. Group 1= STR less than 50%; group 2 = maximum STR ≥ 50%.	Total homocysteine was analysed using HPLC-FD.	Plasma homocysteine levels were 22.5±10 µmol/l in group 1 and 14.1±4 µmol/l in group 2 ($p<0.001$).	Studied concluded that plasma homocysteine levels are associated with impaired STR after fibrinolytic therapy in acute STEMI.
(Ghosh, Khare and Shetty 2009) India	Observe the prevalence of plasma hyperhomocysteinemia in a patient cohort with MI after a 2 week folic acid intervention	N=120 patients with acute MI. Age <40yrs. Control group N=500 age and sex matched. Patients given 2 week intervention of 5 mg folic acid/daily.	Tested for homocysteine 8-10 weeks after MI. Plasma homocysteine measured using a microplate enzyme immunoassay kit.	63 out of 120 patients presented with hyperhomocysteinaemia compared with 9% of the age and sex matched controls. 57% of patients with hyperhomocysteineamias responded to the oral folic acid therapy. The cyanoco-balamin therapy only normalised homocysteine level in 2 out of 12 patients	Study concluded that hyperhomocysteinaemia is common amongst young acute myocardial infarction patients from western India.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Yoldas et al. 2008) Turkey	Investigate the change in hsCRP and homocysteine during the acute period of ischemic stroke and evaluate the relationship between these two biomarkers.	N=40 patients admitted with ischemic stroke. hsCRP and homocysteine measured at the 2nd, 5thand 10th day post stroke. N=40 controls, age and sex matched. Mean age 70±9 years.	Homocysteine determined using HPLC with fluoresce detection, using the Clin Rep Kit.	Patients mean homocysteine levels were 40.6±9.6 µmol/l on the 2nd day, 21.7±11.1 µmol/l on the 5th day and 20.7±9.2 µmol/l on the 10th day. The mean homocysteine level on the control subjects was 11±1.1 µmol/l. The homocysteine levels of the patients were higher than the control subjects at all times ($p<0.01$).	Study concluded that patients with stroke have higher circulating serum hsCRP and homocysteine levels. However although homocysteine was found to be higher, it does not seem to be related to prognosis.
(Kalita et al. 2009) India	Comparison of the risk factors, nature of stroke and outcome of patients with and without hyper-homocysteinemia.	N=198 patents with ischemic stroke (36 female with median age 56 yrs). N=200 healthy controls. Biomarkers measured 1 month after stroke.	Serum B_{12}, folate and homocysteine were determined using ADVIA direct chemiluminscence assay.	Serum homocysteine was elevated in 60.6% and serum B_{12} low in 25.7% and folic acid in 42.1%. Homocysteine levels correlated with serum vitamin B_{12}. Homocysteine levels in stroke patients did not significantly differ from controls.	Study concluded that hyperhomocysteinemia was present in 60.6% of stroke patients which is related to low serum vitamin B_{12} levels. Patients with hyper-homocysteinemia had a better 3-month outcome.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Saposnik et al. 2009) USA	To determine if vitamin therapy reduced the risk of ischemic and hemorrhagic stroke as well as stroke disability.	N=5522 adults (aged 55 years or older) with known CVD; randomised to receive daily combination of 2.5mg folic acid, 50 mg vitamin B_6 and 1 mg of vitamin B_{12} or placebo for 5 years.	Homocysteine was measured using the fluorescence polarization immunoassay.	The mean homocysteine concentration decreased by 2.2 µmol/l in the vitamin therapy group and increased by 0.80 µmol/l in the placebo group.	Lowering of homocysteine with folic acid, vitamins B_6 and B_{12} did reduce the risk of overall stroke, but not the severity of the stroke or disability.
(Osorio et al. 2008) Spain	Investigate the levels of homocysteine during post MI follow-up.	N=127 male patients with acute MI. N= 90 health controls. Bloods taken on day 0, 2, 5, 7, 9 and 11 post MI and compared.	Homocysteine, folate and vitamin B_{12} determined using a competitive chemilusncent immunoassay.	MI patients had higher homocysteine levels on day 0 versus controls. Patients with similar homocysteine levels to controls on day 0 showed significantly higher levels at each time-point post infraction. Patients with significantly higher ($p<0.001$) homocysteine levels than controls on day 0 showed significantly lower levels at time-points post infraction.	Study concluded that different behaviours of homocysteine levels in MI patients might correspond to a history or absence of asymptomatic myocardial ischemia. Further research is required.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Puttonen et al. 2009) Finland	Investigate the association between shift work and cardiovascular disease risk.	N=1543 (712 men and 831 females) young adults recruited as part of Cardiovascular Risk in Young Finns study.	Homocysteine was measured using the microparticle enzyme immunoassay kit.	Mean (SD) homocysteine in males day shift 9.14 (3.27); shift work 8.74 (2.70), p=0.126. Mean homocysteine females day shift 10.84 (4.08), night shift 10.83 (4.48), p=0.955. These relationships persisted after adjustment for age and risk factors such as low socio-economic positions, job strain, smoking, diet, family history of CHD, physical inactivity, alcohol consumption, obesity, homocysteine, C-reactive protein, blood pressure and lipids. In women, no association was found between shift work and carotid atherosclerosis indicators.	Study concludes that the results suggest that shift work accelerates the atherosclerotic process and that the effects of shift work on subclinical atherosclerosis are observed in men already before the age of 40.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Nevado Jr and Imasa 2008) Philippine	To determine the effects of clinical risk factors that are responsible for the occurrence of mortality in myocardial infarction.	N=124 Filipino patients. Composite outcomes of mortality and nonfatal myocardial infarction were measured after 6 months.	Homocysteine levels were determined using the Abbott Imx assay.	Homocysteinemia (>16 µmol/l) is associated with increased mortality and composite outcomes (mortality, nonfatal re-infarction and serious rehospitalisation). No association was detected for the conventional risk factor.	Increased homocysteine levels are associated with mortality and serious nonfatal outcomes in patients with unstable angina and NSTEMI.
(Ranucci et al. 2009) Italy	To investigate whether elevated homocysteine further increases the morbidity and mortality in patients undergoing cardiac surgery on cardiopulmonary bypass (CPB).	N=531 undergoing cardiac operations on CPB.	Total homocysteine was determined using an immuno-fluorescence method.	Elevated homocysteine levels (>15 µmol/l) were observed in 209 patients. Homocysteinaemia was associated with higher mortality and preoperative morbitity (major morbidity, low cardiac output, acute renal failure, mesenteric infarction and thrombo-embolic events). Pre-operative homocysteine levels remained independently associated with hospital mortality.	Study concluded that elevated pre-operative homocysteine levels are independently associated with increased morbidity and mortality particularly in patients undergoing CPB.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Min et al. 2009) Korea	Evaluate the usefulness of baseline homocysteine levels for predicting the risk of short or long-term cardiovascular events after successful coronary stenting.	N=2019 patients with clinical events and normal preprocedural creatine kinase (CK)-MB levels.	Poster-abstract. Methodology not stated.	After multi-variate logistic analysis, homocysteine levels (OR 1.03; 95% CI 1.01-1.05), female sex (OR 1.86; 95% CI 1.31-2.65), hypertension (OR 2.49; 95% CI 1.06-2.10), multivessel diseases (OR 2.21; 95% CI 1.40-3.47), American Heart Association lesion type B2/C (OR 2.53; 95% CI 1.32-4.85) and stent length (OR 2.53; 95% CI 1.32-4.85) were independently associated with peroiprocediral MI.	Study concluded that preprocedural levels of homocysteine were an independent predictor for preprocedural MI and death or MI after successful coronary stenting.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Orhan et al. 2009) Turkey	To evaluate the relationship between homocysteine levels and the development of left ventricular thrombus in acute anterior myocardial infarction patients directed to thrombolytic therapy.	N=95 patients presenting with ST elevated acute anterior MI. Two groups N=14 of thrombosis in the left ventricle following MI or N=65 with no thrombosis.	Total (free plus bound) homocysteine level was determined using HPLC.	Total fasting homocysteine levels (18.24±5.67 mmol/l versus 12.31±3.52 mmol/l $p<0.001$) were significantly higher in patients with left ventricular thrombosis. Higher homocysteine levels ($p=0.04$) were independent predictors of left ventricular thrombus formation.	Study concluded that diabetes mellitus, higher wall motion score index and hyperhomocysteinemia independently increases the risk for the development of left ventricular thrombus formation in patients with acute anterior myocardial infarction following thrombolytic therapy.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Sternic et al. 2009) Serbia	Investigate hyper-homocysteinemia in individuals with established ischemic cerebrovascular disease.	N=55 males and N= 40 females. Mean age 55.6±14.9 years. Established ischemic cerebrovascular disease.	Limited methodology as only published abstract. Homocysteine analysed in plasma.	Mean group homocysteine level 14.3±5.03 µmol/l. Univariate analysis showed higher homocysteine level was associated with increased risk of cognitive decline.	Study indicated association between high plasma homocysteine levels and cognitive decline in patients with established ischemic cerebrovascular disease.
(Hyun et al. 2009) Korea	Investigate association of total plasma homocysteine with arterial stiffness, brachial-artery pulse wave velocity (baPWV), LDL atherogenecity and inflammation.	N=612 healthy men; age range 31-79 years.	Methodology not stated as study is published as a conference abstract.	In all patients total homocysteine concentrations showed positive correlation with age. Negative correlation with folate and vitamin B_{12}. Total homocysteine was associated with baPWV in men with high total homocysteine level (≥13.1 µmol/l, N=153; R=0.258, p=0.001) after adjustments for age, BMI, smoking, drinking, folate and B_{12}. This was not found in patients with low total homocysteine (p=0.478).	Study concluded that enhanced arterial stiffness in hyperhomocysteinemia could be attributed in part to homocysteine related LDL atherogenecity.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Krstevska et al. 2009) Macedonia	Study investigated the levels of homocysteine in healthy subjects and patients with arterial occlusive disease (AOD) and the prevalence of MTHFR, with an aim to show a link.	Healthy controls and patients with AOD. No numbers given as study published as a conference abstract.	Homocysteine methodology not stated. MTHFR polymorphism indentified using PCR with CVD StripAssay as a segment of 12 mutations of the cardiovascular system.	Total homocysteine levels were significantly higher in patients with AOD. Within the Macedonian healthy population, frequency of C allele was dominant then in T allele and was similar in patients. The greatest gene mutations, 45% had the heterozygote gene CT, the homozygote for the wild genotype CC, 42% and genotype TT 13%. There was no significant difference of gene mutations as risk factor for AOD. Homocysteine levels of healthy subjects with different genotypes of $MTHFR_{(C677T)}$ showed significantly higher values of TT and CT genotype compared to CC genotype.	Study concluded that there was no correlation of $MTHFR_{(C677T)}$ polymorphism with AOD.

Reference & Country	Aim	Participants	Methodology	Results	Conclusion
(Agoston-Coldea et al. 2009) France	Investigated the relationship between homocysteine and apolipoproteins A-1 and B concentrations in patients with prior myocardial infractions (MI).	N=208 patients (100 men and 108 women); aged 60.08±10.43 years. 2 groups: 104 patients with prior MI and 104 without CAD.	Methodology not stated as study is published as a conference abstract	Patients with prior MI had higher mean values of plasma homocysteine than the control patients (18.98±4.72 versus 14.09±3.32 µmol/l, p≤0.001). Study results showed a positive correlation between plasma homocysteine levels and the severity of coronary lesions (R=0.765, p<0.05).	Study concluded that plasma homocysteine is positively correlated with the number of injured coronary vessels. Study suggests homocysteine and apoB/apoA-1 could be used as biomarkers of cardiovascular risk.
(Naono et al. 2009) Japan	Investigated the association plasma homocysteine levels with long-term major adverse cardiovascular events (MACEs).	N=231 patients with previous percutaneous coronary intervention (PCI).	Homocysteine in plasma measured using HPLC.	An univariate analyses by a Cox proportional hazards regression model showed that plasma homocysteine level was not associated with the primary (hazard ratio [HR] 1.13, 95% CI 0.41-3.08, p=0.82) and secondary (HR 1.60, 95% CI 0.75-3.42, p=0.23) end points.	Study concluded that plasma homocysteine levels appear to be unrelated to recurrent angina, new MI and long-term MACE within coronary artery disease patients with previous PCI.

APPENDIX 2

A. HI Study QMU Ethics Approval

Queen Margaret University
EDINBURGH

Julie Murdoch
Research Student
Dietetics, Nutrition and Biological Sciences
School of Health Sciences

Linda Graham
Registry Officer
Queen Margaret University College
Clerwood Terrace
Edinburgh EH12 8TS

Tel: 0131 317 3219
Email: lgraham@qmuc.ac.uk

29 March 2007

Dear Julie

Ethical Approval – The effect of alcohol toxicology in the form of binge drinking and other drinking patterns, on biomarkers of cardiovascular disease risk and nutritional uptake in young adults

Thank you for your response dated 22 February 2007 to the letter I sent you following consideration of your application by the Research Ethics Committee.

Alanah Kirby, Convener of the Committee, has reviewed your response to the points you were required to address, and has confirmed that she is happy to take Convener's Action to grant full ethical approval for your research.

A standard condition of this ethical approval is that you are required to notify the Committee, in advance, of any significant proposed deviation from the original protocol. Reports to the Committee are also required once the research is underway if there are any unexpected results or events that raise questions about the safety of the research. Notification of completion of the study is also required – please find the appropriate form for this enclosed.

We would like to thank you for your co-operation and wish you well with your project.

Yours sincerely,

Linda Graham

Linda Graham
Secretary to the Research Ethics Committee

Cc Dr Jan Gill, Supervisor

B. HI Study QMU Ethics Approval Amendment

Queen Margaret University

EDINBURGH

Julie Murdoch
Research Student
Nursing
School of Health Sciences

Linda Graham
Registry Officer
Queen Margaret University
Queen Margaret University Drive
Musselburgh
East Lothian EH21 6UU

Tel: 0131 474 0000
Email: lgraham@qmu.ac.uk

29 April 2009

Dear Julie

Ethical Approval – The effect of toxicology in the form of different alcohol drinking patterns on biomarkers of cardiovascular disease risk

Thank you for your submission dated 24 March 2009 notifying the Research Ethics Committee of a change to your previously approved protocol for the above project.

Professor Marie Reid, Convener of the Committee, has reviewed your submission, and has confirmed that she is happy to take Convener's Action to grant full ethical approval for your research.

We would like to thank you for your co-operation and wish you well with your project.

Yours sincerely

pp.

Linda Graham
Secretary to the Research Ethics Committee

Cc Dr Jan Gill, Supervisor

C. HI Study Edinburgh Napier University Approval

www.napier.ac.uk

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Julie Murdoch
Research Student
Dietetics, Nutrition and Biological Sciences
School of Health Sciences
Queen Margaret University
Clerwood Terrace
Edinburgh EH12 8TS

29 May 2007

Dear Julie

APPLICATION FOR ETHICAL APROVAL FOR A RESEARCH PROJECT – The effect of alcohol toxicology in the form of binge drinking patterns, on biomarkers of cardiovascular disease risk and nutritional uptake in young adults.

I am pleased to confirm that Ethical Approval and Access for the above project has now been granted.

If you have any questions please do not hesitate to contact me, or Dr Maureen Macmillan (m.Macmillan@napier.ac.uk tel. 0131 455 5663.

Yours sincerely

LA Laid

Lesley Laidlaw
Assistant Faculty Manager
Faculty of Health, Life & Social Sciences
Email: l.Laidlaw@napier.ac.uk
Tel: 0131 455 5622

INVESTOR IN PEOPLE

NAPIER UNIVERSITY
EDINBURGH

D. ADI Study NHS Ethics Approval

Lothian NHS Board

Deaconess House
148 Pleasance
Edinburgh
EH8 9RS
Telephone 0131 536 9000
Fax 0131 536 9009
www.nhslothian.scot.nhs.uk

NHS Lothian

Lothian Local Research Ethics Committee 02
Telephone: 0131 536 9061
Facsimile: 0131 536 9346

20 July 2007

Miss Julie M. Murdoch
PhD Student
Queen Margaret University College
School of Health Sciences
Corstorphine Campus,
Clerwood Terrace
Edinburgh
EH12 8TS

Dear Miss Murdoch

Full title of study: **Effect of alcohol toxicology in the form of different alcohol drinking patterns on biomarkers of cardiovascular disease (CVD) risk.**

REC reference number: **07/S1102/23**

Thank you for your letter of 26 July 2007, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered at the meeting of the Sub-Committee of the REC held on 12 July 2007.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	5.3 (2)	14 May 2007
Investigator CV		15 May 2007
Protocol	1	15 May 2007
Covering Letter		15 May 2007

Questionnaire: for In-Patient Participants within the Alcohol Problems Unit		
GP/Consultant Information Sheets	2	
Participant Information Sheet: Participant	2	
Participant Consent Form: Participant	2	
Response to Request for Further Information		26 July 2007
Statement of Indemnity Arrangements		11 September 2006
Supervisor's CV		16 May 2007
Letter from Statistician		04 May 2007

R&D approval

All researchers and research collaborators who will be participating in the research at NHS sites should apply for R&D approval from the relevant care organisation, if they have not yet done so. R&D approval is required, whether or not the study is exempt from SSA. You should advise researchers and local collaborators accordingly.

Guidance on applying for R&D approval is available from http://www.rdforum.nhs.uk/rdform.htm.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

Feedback on the application process

Now that you have completed the application process you are invited to give your view of the service you received from the National Research Ethics Service. If you wish to make your views known please use the feedback form available on the NRES website at:

https://www.nresform.org.uk/AppForm/Modules/Feedback/EthicalReview.aspx

We value your views and comments and will use them to inform the operational process and further improve our service.

07/S1102/23	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Professor Peter Hayes
Chair
Email: lyndsay.baird@lhb.scot.nhs.uk

Enclosures: *Standard approval conditions*
Site approval form

Copy to: Miss Julie M. Murdoch, Queen Margaret University College

E. ADI Study Research and Development Approval

University Hospitals Division

Queen's Medical Research Institute
47 Little France Crescent, Edinburgh, EH16 4TJ

NHS Lothian

HAC/SM/approval/2e/3/3b

27th September 2007

Miss Julie Murdoch
Queen Margaret University
School of Health Sciences
Corstorphine Campus,
Clerwood Terrace
Edinburgh
EH12 8TS

RESEARCH & DEVELOPMENT OFFICE
Room E1.12

Tel: 0131 242 3330
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Email:
R&DOffice@luht.scot.nhs.uk

Director:
Professor Heather A Cubie

R&D Governance Manager
DrTina McLelland

PA to Professor Cubie & Dr McLelland:
Mrs Jill Kelly

Commercial Research Manager:
Dr Douglas Young

Research Manager Capacity & Capability:
Dr Janet Hanley

Research Governance Co-ordinator:
Mrs Susan Shepherd

Information & Knowledge Manager
Miss Heather Coupar

AHP Research & Development Facilitator:
Dr Colette Fulton

Accountant:
Ms Sheevaun McIntyre

Assistant Accountant:
Mr Neil McLean

Trial Support Officer:
Ms Dorothy Aitken

Office Manager:
Mrs Glynis Omond

Administrative Assistant:
Ms Sandra Muir

St John's – Administrator:
Mrs Anne Addison

Dear Miss Murdoch

MREC No:	**N/A**
CRF No:	**N/A**
LREC No:	**07/S1102/23**
R&D ID No:	**2007/P/PSY/19**
Title of Research	Effect of alcohol toxicology in the form of different alcohol drinking patterns on biomarkers of cardiovascular disease (CVD) risk.
Protocol No/Acronym:	**N/A**

The above project has undergone an assessment of risk to NHS Lothian and review of resource and financial implications. I am satisfied that all the necessary arrangements have been set in place and that all Departments contributing to the project have been informed.

I note that this is a single centre study sponsored by **Queen Margaret University.**

Use of Tissue or Samples

- The study involves the use of patient tissue or samples. You must be familiar with NHS Lothian's Tissue Policy and abide by its conditions and also with all regulations in place at the time. Approval is subject to the prevailing legal requirements.
- Approval for the use of tissue is restricted to the protocol associated with this application, but may include additional collaborators within University of Edinburgh. Collaborators who are not named in the original protocol require to be notified to local REC.
- If material is to be transferred to academic collaborators outwith University of Edinburgh or to any commercial entity then a Material Transfer Agreement must be obtained from the R&D Office and signed by all relevant parties prior to transfer of the material. Such collaborations must be fully discussed with the R&D Office and management approval is only effective once this is in place.

- I note that additional samples will be taken for the study and that this will be done with the patient's explicit consent.

On behalf of the Chief Executive and Medical Director, I am happy to grant management approval from NHS Lothian to allow the project to commence, subject to the approval of the appropriate Research Ethics Committee(s) having also been obtained. You should note that any substantial amendments must be notified to the relevant Research Ethics Committee and to R&D Management with approval being granted from both before the amendments are made.

Please note that under Section A, Q35, NHS Lothian provides indemnity for negligence for NHS and Honorary clinical staff for research associated with their clinical duties. It is not empowered to provide non-negligent indemnity cover for patients. NHS Lothian does not provide indemnity against negligence for healthy volunteer studies. This is the personal responsibility of both NHS and honorary employees and is usually arranged with a medical defence organisation or through the University of Edinburgh.

This letter of approval is your assurance that NHS Lothian is satisfied with your study. As Chief Investigator or local Principal Investigator, you should be fully committed to your responsibilities within the Research Governance Framework for Health and Community Care, an extract of which is attached to this letter.

Yours sincerely

Heather A Cubie

Professor Heather A Cubie
R&D Director

Enc	Research Governance Certificate	☑ (to be signed and returned)
	NRR authorisation	☑ (to be signed and returned)
	Tissue Policy (if applicable)	☑
	MTA (if applicable)	☐ (to be signed and returned by the recipient of Tissue)

Copies *Administrators, Research Ethics Committee*
Dr Jonathan Chick, Consultant, Royal Edinburgh Hospital

F. HI Study Email and Poster Advertisement

THIS PROJECT HAS ETHICAL APPROVAL

!!!!!!!ME!!!!!!

WHAT KIND OF DRINKER ARE YOU?????

Would you like to be part of the biomedical PhD research project: "The effect of alcohol toxicology in the form of different alcohol drinking patterns on biomarkers of cardiovascular disease (CVD) risk"

DRINKERS & NON-DRINKERS WELCOME
MALE & FEMALES AGED 18-50 YRS

If you would like to take part and get more information on this project, please contact me on the numbers/e-mail below.

Julie Murdoch
Tel: (0131) 474 0000
Mobile: 07796947611
E-mail: jmurdoch@qmu.ac.uk

G. HI Study Information Sheet - Version 1

Queen Margaret University

EDINBURGH

Information Sheet

My name is Julie Murdoch and I am a PhD student from the School of Health Sciences at Queen Margaret University in Edinburgh. As part of my PhD degree, I am undertaking a research project entitled: **"The effect of alcohol toxicology in the form of different alcohol drinking patterns on biomarkers of cardiovascular disease (CVD) risk".**

This study will investigate the levels of a naturally occurring compound in blood, known as homocysteine (Hcy). Increasing levels of homocysteine have been linked to increasing CVD risk. Different alcohol drinking patterns may also be relevant and for this reason the study will also measure; an alcohol consumption biomarker; two vitamins from the B-vitamin family and identify the presence of two forms of a gene which influences Hcy metabolism in blood.

The findings of the project will be valuable because levels of Hcy have not been analysed in individuals who consume alcohol in a non-chronic pattern. The data from this study will be compared to data gathered within an alcohol rehabilitation unit.

I am looking for volunteers to participate in the project. Please see table 1 which details the inclusion (people who **can** take part) and exclusion (people who **can not** take part) criteria.

Table 1: Inclusion and Exclusion Criteria

Inclusion Criteria	Exclusion Criteria
Men and women aged 18-50yrs	Pregnant
In good health	Trying to become pregnant
	Breast-feeding
	History of cardiovascular disease
	History of cancer
	Diabetes mellitus
	Gastric or duodenal ulcers
	Liver or gallbladder disease
	Known history of blood borne viruses

If you agree to participate in the study, you will be asked to follow the timetable which is shown in table 2. Female volunteers will be asked to take a section of the questionnaire home, to answer questions relating to their menstrual history, in private and return it in the pre-paid envelope provided.

Table 2: Study Timetable for Participants

Timetable	What is required of participant
Questionnaire and Introduction Meeting at a place and time most convenient to the participant.	You will be asked to complete a questionnaire and will be given a urine sample container and a 7-day alcohol and diet diary. Female participants will be asked to return the questionnaire in a pre-paid envelope.
Day 1 (Baseline) at QMU	You will be asked to bring to the meeting a waking urine sample, and the completed alcohol and diet diary. Fasting* blood sample will be taken. You will be given another 7-day diet and alcohol diary. Appointment for 3 months time will be made to return to QMU for blood and urine sampling.
Day 2 (Three months from date of baseline sampling) at QMU	You will be asked to bring to the meeting a waking urine sample, and the completed alcohol and diet diary. Fasting* blood sample will be taken. You will be given another 7-day diet and alcohol diary. Appointment for 3 months time will be made to return to QMU for blood and urine sampling.
Day 3 (Six Months from date of baseline sampling) at QMU	You will be asked to bring to the meeting a waking urine sample, and the completed alcohol and diet diary. Final fasting* blood sample will be taken.

* You should not eat 12 hours before the time of your blood sampling appointment, for example if your appointment is at 8am you should eat nothing after 8pm the previous night. Once the blood sample has been taken you will be offered a soft drink and biscuits before you leave QMU.

The blood that you provide will be analysed using various scientific techniques for the levels of Hcy; an alcohol consumption biomarker; vitamins and 2 forms of a gene which is important to Hcy metabolism. The urine that you provide will be used to develop an analytical method for identifying Hcy in urine, thus hopefully providing a less invasive method for use in future studies.

If you are found to have the second form of the gene which influences Hcy metabolism, you will be informed of this result and will not be included in the study. This is because the second form of this gene can affect the way the body metabolises Hcy, which will result in anomalies within the data. I will provide you with a letter of this finding to take to your general practioner, who will advise you of the medical aspects of this finding, and he/she may advise that you increase your folate intake. All information regarding participant's genetic information will be destroyed when the study is finished.

Since your participation will involve you travelling to Queen Margaret University, you will be reimbursed for out-of-pocket travel expenses.

All data will be confidential and be anonymised. Your name will be replaced with a participant number, and it will not be possible for you to be identified in any reporting of the data gathered. Once my thesis has been published all original questionnaires will be destroyed.

The results of this study will be included in my PhD thesis and may be published in a scientific journal or presented at a scientific conference.

If you would like to contact an independent person, who knows about this project but is not involved in it, you are welcome to contact Dr Mary Warnock. Her contact details are given in table 3.

If you have read and understood this information sheet, any questions you had have been answered, and you would like to be a participant in the study, please read and sign the consent form.

Table 3: Contact details of Researcher and Independent Advisor

	Contact detail of the Researcher	***Contact details of the Independent Advisor***
Name	Julie Murdoch	Dr. Mary Warnock
Address	PhD Student, Nursing School of Health Sciences Queen Margaret University Queen Margaret University Drive Edinburgh EH21 6UU	Lecturer Dietetics, Nutrition & Biological Sciences School of Health Sciences Queen Margaret University Queen Margaret University Drive Edinburgh EH21 6UU
E-mail	jmurdoch@qmu.ac.uk	mwarnock@qmu.ac.uk
Telephone	0131 474 0000	0131 474 0000
Mobile/Fax	07796947611	0131 474 0001

H. HI Study Information Sheet - Version 2

Queen Margaret University

EDINBURGH

Information Sheet

My name is Julie Murdoch and I am a PhD student from the School of Health Sciences at Queen Margaret University in Edinburgh. As part of my PhD degree, I am undertaking a research project entitled: **"The effect of alcohol toxicology in the form of different alcohol drinking patterns on biomarkers of cardiovascular disease (CVD) risk".**

This study will investigate the levels of a naturally occurring compound in blood, known as homocysteine (Hcy). Increasing levels of homocysteine have been linked to increasing CVD risk. Different alcohol drinking patterns may also be relevant and for this reason the study will also measure; an alcohol consumption biomarker; two vitamins from the B-vitamin family and identify the presence of two forms of a gene which influences Hcy metabolism in blood.

The findings of the project will be valuable because levels of Hcy have not been analysed in individuals who consume alcohol in a non-chronic pattern. The data from this study will be compared to data gathered within an alcohol rehabilitation unit.

I am looking for volunteers to participate in the project. Please see table 1 which details the inclusion (people who **can** take part) and exclusion (people who **can not** take part) criteria.

Table 1: Inclusion and Exclusion Criteria

Inclusion Criteria	Exclusion Criteria
Men and women aged 18-50yrs	Pregnant
In good health	Trying to become pregnant
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	Gastric or duodenal ulcers
	Liver or gallbladder disease
	Known history of blood borne viruses

If you agree to participate in the study, you will be asked to follow the timetable which is shown in table 2. Female volunteers will be asked to take a section of the questionnaire home, to answer questions relating to their menstrual history, in private and return it in the pre-paid envelope provided.

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Day 3 (Six Months from date of baseline sampling) at QMU	You will be asked to bring to the meeting a waking urine sample, and the completed alcohol and diet diary. Final fasting* blood sample will be taken.

* You should not eat 12 hours before the time of your blood sampling appointment, for example if your appointment is at 8am you should eat nothing after 8pm the previous night. Once the blood sample has been taken you will be offered a soft drink and biscuits before you leave QMU.

The blood that you provide will be analysed using various scientific techniques for the levels of Hcy; an alcohol consumption biomarker; vitamins and 2 forms of a gene which is important to Hcy metabolism. The urine that you provide will be used to develop an analytical method for identifying Hcy in urine, thus hopefully providing a less invasive method for use in future studies.

If you are found to have the second form of the gene which influences Hcy metabolism, you will be informed of this result by letter and you will be asked to sign a consent form, if you would still like your data to be analysed. I will provide you with a letter of this finding to take to your general practioner, who will advise you of the medical aspects of this finding, and he/she may advise that you increase your folate intake. All information regarding participant's genetic information will be destroyed when the study is finished.

Since your participation will involve you travelling to Queen Margaret University, you will be reimbursed for out-of-pocket travel expenses.

All data will be confidential and be anonymised. Your name will be replaced with a participant number, and it will not be possible for you to be identified in any reporting of the data gathered. Once my thesis has been published all original questionnaires will be destroyed.

The results of this study will be included in my PhD thesis and may be published in a scientific journal or presented at a scientific conference.

If you would like to contact an independent person, who knows about this project but is not involved in it, you are welcome to contact Dr Mary Warnock. Her contact details are given in table 3.

If you have read and understood this information sheet, any questions you had have been answered, and you would like to be a participant in the study, please read and sign the consent form.

Table 3: Contact details of Researcher and Independent Advisor

	Contact detail of the Researcher	***Contact details of the Independent Advisor***
Name	Julie Murdoch	Dr. Mary Warnock
Address	PhD Student, Nursing School of Health Sciences Queen Margaret University Queen Margaret University Drive Edinburgh EH21 6UU	Lecturer Dietetics, Nutrition & Biological Sciences School of Health Sciences Queen Margaret University Queen Margaret University Drive Edinburgh EH21 6UU
E-mail	jmurdoch@qmu.ac.uk	mwarnock@qmu.ac.uk
Telephone	0131 474 0000	0131 474 0000
Mobile/Fax	07796947611	0131 474 0001

I. HI Study Consent Form

Queen Margaret University

EDINBURGH

Consent Form

"The effect of alcohol toxicology in the form of different alcohol drinking patterns on biomarkers of cardiovascular disease risk".

I have read and understood the information sheet and this consent form. I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in this study.

I understand that I have the right to withdraw from this study at any stage without giving any reason.

I agree to participate in this study.

Name of participant: _____

Signature of participant: _____

Signature of researcher: _____

Date: _____

Contact details of the researcher

Name of researcher: Julie Murdoch
Address: PhD Student, Nursing, School of Health Sciences
Queen Margaret University
Queen Margaret University Drive, Edinburgh, EH21 6UU
Email: jmurdoch@qmuc.ac.uk
Telephone: 0131 474 0000
Mobile: 07796947611

J. ADI Study Information Sheet

Queen Margaret University

EDINBURGH

Patient Information Sheet

Study Title: **"The effect of alcohol toxicology in the form of different alcohol drinking patterns on biomarkers of cardiovascular disease (CVD) risk".**

My name is Julie Murdoch and I am a PhD student from the School of Health Sciences at Queen Margaret University in Edinburgh. As part of my PhD degree, I am undertaking a research project. I would like to invite you to take part in the above research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to other people about the study if you wish.

Part 1 tells you the purpose of this study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study. Please ask me or the independent advisor or the medical staff of the Alcohol Problems Unit (APU) if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part.

Part 1

This study will investigate the levels of a naturally occurring compound in blood, known as homocysteine (Hcy). Increasing levels of homocysteine have been linked to increasing CVD risk. Different alcohol drinking patterns may also be relevant and for this reason the study will also measure; an alcohol consumption biomarker; two vitamins from the B-vitamin family and identify the presence of two forms of a gene which influences Hcy metabolism in blood. This research project will form a chapter within my PhD thesis.

The finding of the project will be valuable as the results from this study will be compared to results from another study I am running which looks at levels of the above biomarker's within abstainers, binge drinkers and occasional drinkers.

You are being invited to take part in this study as you are: male or female aged 18-50 years, are an in-patient of the Alcohol Problems Unit (APU) within the Royal Edinburgh Hospital or an out-patient of the Alcohol Problem Service and are taking the prescribed medication chlordiazepoxide.

It is up to you to decide to take part. I will describe the study and go through this information sheet which I will then give to you. I will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

If you agree to participate in the study, you will be asked to follow the timetable which is shown in table 2. Female volunteers will be asked to complete a section of the questionnaire in private, relating to questions about their menstrual history. It is necessary to ask female participants questions regarding their menstrual, contraceptive and menopausal history as they have an effect on the level of Hcy in the blood.

Table 2: Study Timetable for Participants

Timetable	What is required of participant
Questionnaire and Introduction Meeting at the Ritson Clinic or 35 Morningside Park	You will be asked to complete a questionnaire. Female participants will be asked to complete a section of the questionnaire in private.
Day 1 (Baseline) at APU or 35 Morningside Park	Fasting* blood sample will be taken. Waking urine sample
Day 3 at Ritson Clinic or 35 Morningside Park	Fasting* blood sample will be taken. Waking urine sample

* You should not eat 12 hours before the time of your blood sampling appointment, for example if your appointment is at 8am you should eat nothing after 8pm the previous night. Once the blood sample has been taken you will be offered a soft drink and biscuits.

On the day of your admission to the APU routine blood tests were carried out, one of these tests was for the level of folate and vitamin B_{12} within your body. The results of that specific test will be made available to me, the researcher to include with the other data you have provided for this study. Also if you are being prescribed B vitamin supplements orally or intravenously as part of your clinical care package then the dose of the supplements will also be made available to the researcher.

The blood that you provide will be analysed using various scientific techniques for the levels of Hcy; an alcohol consumption biomarker; vitamins and 2 forms of a gene which is important to Hcy metabolism. The urine that you provide will be used to develop an analytical method for identifying Hcy

in urine, thus hopefully providing a less invasive method for use in future studies.

If you are found to have the second form of the gene which influences Hcy metabolism, you will be informed of this result and will not be included in the study analysis. I will provide you with a letter of this finding to take to your general practitioner (GP), who will advise you of the medical aspects of this finding, and he/she may advise that you increase your folate intake, such as increasing your fruit and vegetable intake. All information regarding participant's genetic information will be destroyed when the study is finished.

I cannot promise that your participation in this study will help you, but the information I get from this study may help improve people's awareness of alcohol related health problems.

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Your participation in this study will be kept confidential and I will follow ethical and legal practise and all information about you will be handled confidentially. The details are included in Part 2.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Part 2

Sometimes new information becomes available. If this happens the researcher will tell you and discuss whether you should continue in the study. If you decide not to carry on, the researcher will make arrangements for your care to continue. If you decide to continue in the study the researcher may ask you to sign an updated consent form. Also if new information becomes available the researcher might consider that you should withdraw from the study. The researcher will explain the reasons and arrange for your care to continue. If the study is stopped for any reason the researcher will inform you and arrange for your care to continue.

If you decide to withdraw from the study for any reason, all information and samples that you provide will be destroyed and will be not used in any data analysis.

If you have a concern about any aspect of this study, you should ask to speak to the researcher or independent advisor who will do their best to answer your questions (please see table 3 for contact details). If you remain

unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

Table 3: Contact details of Researcher and Independent Advisor

	Contact detail of the Researcher	***Contact details of the Independent Advisor***
Name	Julie Murdoch	Dr. Mary Warnock
Address	PhD Student, Nursing, School of Health Sciences Queen Margaret University Queen Margaret University Drive Musselburgh EDINBURGH EH21 6UU	Lecturer, Dietetics, Nutrition & Biological Sciences School of Health Sciences Queen Margaret University Queen Margaret University Drive Musselburgh EDINBURGH EH21 6UU
E-mail	jmurdoch@qmu.ac.uk	mwarnock@qmu.ac.uk
Telephone	(0131) 474 0000	(0131) 474 0000
Mobile/Fax	07796947611/(0131) 474 0001	(0131) 474 0001

In the event that something goes wrong and you are harmed during the research and this is due to the researcher's negligence then you may have grounds for a legal action for compensation against Queen Margaret University, but you may have to pay your legal costs. The normal NHS complaints mechanisms will still be available to you.

All information which is collected about you during the course of the research will be kept strictly confidential and any information about you which leaves the hospital will have your name removed so that you cannot be identified.

Your General Practitioner (GP) will be informed via letter of your participation within this study. Your GP will also be sent a copy of this information sheet.

The biological samples that you provide will be new samples, in excess of clinical care samples and will be stored under participant numbers, which will only be identifiable to the researcher. The samples will be analysed within QMU laboratories and biochemistry laboratories at the Edinburgh Royal Infirmary. All biological samples will be destroyed at the end of the study.

The data that you provide will be secured within a locked cabinet which is only accessible by the researcher. All data will be identifiable by a unique participant number. All data will be destroyed when the study is complete. You will be contacted via letter if you are found to carry the 2nd form of the gene which affects Hcy metabolism, which you are advised to take to your GP. None of the biological samples or data that you provide will be transferred out of the UK.

The results of this study be included in my PhD thesis and may be published in a scientific journal or presented at a scientific conference.

Queen Margaret University is funding this PhD research study. The doctor in charge of your care is **not** being paid for including you in this study.

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights wellbeing and dignity. This study has been reviewed and given favourable opinion by Lothian Research Ethics Committee.

K. ADI Study Consent Form

Centre Number: APU-1
Study Number: 07/S1102/23
Patient Identification Number for this Study:

CONSENT FORM

Title of Project: **Effect of alcohol toxicology in the form of different alcohol drinking patterns on biomarkers of cardiovascular disease (CVD) risk**

Name of Researcher: Julie M. Murdoch

Please Initial Box

1. I confirm that I have read and understood the information sheet dated …….. (version 2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from Queen Margaret University, from regulatory authorities or from the NHS trust where it is relevant to my taking part in this research, I give the permission for these individuals to have access to my records.

4. I agree to my GP being informed of my participation in the study.

5. I agree for genetic analysis to be undertaken on the blood samples which I provide as part of the above study.

6. I agree to take part in the above study.

Name of Patient	Date	Signature
Name of Person taking consent	Date	Signature

When completed, 1 for patient; 1 for researcher site file 1 (original) to be kept in medical notes.

L. HI Study Questionnaire

Queen Margaret University
EDINBURGH

Data Sheet For Participants of:
"The effect of alcohol toxicology in the form of different alcohol drinking patterns on biomarkers of cardiovascular disease (CVD) risk."
Study

Participant Information

Participant Number:

Date of Birth:

Today's Date:

Baseline Sampling Date (To be completed by Researcher):

3 month Sampling Date (To be completed by Researcher):

6 month Sampling Date (To be completed by Researcher):

Please complete each question with a tick in the appropriate box.

Q1. Occupation	QMU Student	QMU Staff	Other University Student	Other University Staff	Research Laboratory Staff
What is your occupation?	○	○	○	○	○

Q2. Sex	Male	Female
What is your Gender?	○	○

Q3. Smoking	Yes	No
Are you a Smoker?	○	○

Q4. Age	18-25 yrs	26-50 yrs
What is your age group?	○	○

Q5. Drinking Pattern	Yes	No
Do you <1-2 alcoholic drinks per year?	○	○

If you answered NO to Question 5, please go to question 6

Q6. Drinking Pattern	Daily	Weekly	Monthly
How frequently to you consume alcohol?	○	○	○
How much alcohol do you consume at one session? (1 unit = ½ pint of beer, or small glass of wine or 1 measure of spirit)			
Would you drink more than >6-8 units of alcohol in one session?	**Yes**	**No**	
	○	○	

If you are male, thank you for taking the time to complete this questionnaire, I would like to confirm that this data will remain confidential and anonymous. Your participation is very much appreciated.

If you are female, can use please take the time to complete Question 7, 8 and 9 on page 3 at home and return it in the pre-paid envelope.

Queen Margaret University
EDINBURGH

Questionnaire For Female Participants of: "The effect of alcohol toxicology in the form of different alcohol drinking patterns on biomarkers of cardiovascular disease (CVD) risk." Study

Participant Number:

Q7. Menstrual Cycle	Yes	No
Are your periods regular?	○	○
When did your last period begin?		

Q8. Contraceptive Pill	Yes	No
Do you take the contraceptive pill?	○	○
What is the brand name your contraceptive pill?		

Please answer Question 9, if appropriate.

Q9. Menopausal Status	Yes	No
Do you consider yourself to be menopausal?	○	○
Do you use HRT?	○	○
How long have you been using HRT?		

Thank you for taking the time to complete this questionnaire, I would like to confirm that this data will remain confidential and anonymous. Your participation is very much appreciated.

Please return this questionnaire in the pre-paid envelope provided.

M. Diet Diary

Queen Margaret University
EDINBURGH

7 DAY FOOD & ALCOHOL DIARY

Participant No : _____
Diet Diary No: _____
Start Date: _____
End Date: _____

During the 7 days of this study you must keep a food and alcohol diary. It is therefore important that the information you record is as accurate as possible. The food diary should be kept for 7 complete days.

Please record food and alcohol consumption in the diary for 7 consecutive days.

Guidance

- Start each day on a new page
- Record the day, date and time
- Attach extra sheets to the back if required

HELPFUL HINTS FOR COMPLETING YOUR FOOD DIARY

- Record *everything* that you eat and drink
- Record what you eat and drink as close as possible to the time you consume it
- Be as accurate as possible when describing the foods you have eaten
- Think of each meal in terms of individual food items
  For example,
  A sandwich may consist of bread, spread, cheese & tomato
  A cup of coffee may also contain milk and sugar
- Provide brand names whenever possible
- State whether foods are homemade or readymade
- Include cooking methods used. E.g. grilling, frying, boiling

PORTION SIZES

- Where possible record the actual weight of the food eaten
- Measure vegetables in terms of tablespoons
- For small fruit such as grapes/ dried fruit, estimate in terms of a cup or handful
- Measure cooked rice, pasta and noodles in terms of tablespoons
- Soups, salads and cereals can be estimated as a small, medium or large bowl
- State the number of slices, the type of bread and whether thin, medium or thick slices are eaten
- Fluids can be estimated as a small medium or large glass unless the specific volume is known

Date: _____

TIME (AM/PM)	FOOD DESCRIPTION	PORTION SIZE/ QUANTITY

N. ADI Study Questionnaire

Queen Margaret University
EDINBURGH

Questionnaire For Patient Participants Within The Alcohol Problems Service

Participant Information

Participant Number:

Date of Birth:

Today's Date:

Baseline (Day 1) Sampling Date (To be completed by Researcher):

Day 3 Sampling Date (To be completed by Researcher):

Please complete each question with a tick in the appropriate box.

Q1. Sex	Male	Female
What is your Gender?	○	○

Q2. Smoking	Yes	No
Are you a Smoker?	○	○

Q3. Age	18-25 yrs	26-50 yrs
What is your age group?	○	○

Q4. Alcohol Dependence	<1 yr	>2 yrs	>5 yrs	>10 yrs	>20 yrs
When did you first make contact with the Alcohol Problems Unit?	○	○	○	○	○

Q5. Type of Alcohol	Wine	Spirit	Cider	Beer
What type of alcohol did you consume?	○	○	○	○

Q6. Drinking Frequency	Daily	Weekly	Monthly
How frequently did you consume alcohol in the last year?	○	○	○

Q7. Time since last alcoholic drink	
How many days since you last consumed alcohol?	

Q8. Drinking in Previous Month	
In the previous month how many days did you drink consume alcohol?	

Q9. Drinking Volume	
How much alcohol did you typically consume in a day when you were drinking? (1 unit = ½ pint of beer, or small glass of wine or 1 measure of spirit)	

If you are male, thank you for taking the time to answer these question's, I would like to confirm that this data will remain confidential and anonymous. Your participation is very much appreciated.

If you are female, can use please take the time to complete, in private Question 10, 11 and 12 on page 3.

Queen Margaret University

EDINBURGH

Questionnaire For Female In-Patient Participants Within The Alcohol Problems Unit

Participant Number:

Please answer each question with a tick in the appropriate box

Q10. Menstrual Cycle	Yes	No
Are your periods regular?	○	○
When did your last period begin?		

Q11. Contraceptive Pill	Yes	No
Do you take the contraceptive pill?	○	○
What is the brand name your contraceptive pill?		

Please answer Question 12, if appropriate.

Q12. Menopausal Status	Yes	No
Do you consider yourself to be menopausal?	○	○
Do you use HRT (Hormone Replacement Therapy)?	○	○
How long have you been using HRT (Hormone Replacement Therapy)?		

Thank you for taking the time to complete this questionnaire, I would like to confirm that this data will remain confidential and anonymous. Your participation is very much appreciated.

O. Alcohol Manufacturers (AM) Method Spreadsheet

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Smirnoff Ice & Bliss	Ready to Drink (RTD)	5.5	275	Bottle	1.5	8	12
WKD; Bacardi Breezer; Diet Bacardi; Reef; Archers Aqua & Other Average RTD	Ready to Drink (RTD)	5	275	Bottle	1.4	8	11.2
Red Square	Ready to Drink (RTD)	5.1	275	Bottle	1.4	8	11.2
Mudshake; Chekov; Bailey's Glide & VK Vodka Kick	Ready to Drink (RTD)	4	275	Bottle	1.1	8	8.8

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol Content (g)/Drink
Bailey's	Liqueur	17	50	Glass	0.9	8	7.2
Southern Comfort	Liqueur	37	50	Glass	1.9	8	15.2
Tia Maria	Liqueur	26.5	50	Glass	1.3	8	10.4
Pimms No1	Liqueur	25	50	Glass	1.3	8	10.4
Archers Aqua	Liqueur	23	50	Glass	1.2	8	9.6
Malibu	Liqueur	21	50	Glass	1.1	8	8.8
Contreau & Drambuie	Liqueur	40	50	Glass	2.0	8	16
Tia Lusso	Liqueur	17	50	Glass	0.9	8	7.2
Tequila	Liqueur	38	25	Shot Glass	1.0	8	7.6
Sambuca	Liqueur	42	25	Shot Glass	1.1	8	8.4
Jagermeister	Liqueur	35	25	Shot Glass	0.9	8	7
Apple Sourz	Liqueur	15	25	Shot Glass	0.4	8	3
After-Shock	Liqueur	40	25	Shot Glass	1.0	8	8
Absinthe	Liqueur	60	25	Shot Glass	1.5	8	12

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Smirnoff Red Vodka; Gordon's Gin; Bacardi; Glen Vodka; London Gin Distiller's Vodka; Vladivar Classic Vodka; Chekov & Other Average Light Spirit	Light Spirit	37.5	25	Small Single	0.9	8	7.2
Smirnoff Red Vodka; Gordon's Gin; Bacardi; Glen Vodka; London Gin Distiller's Vodka; Vladivar Classic Vodka; Chekov & Other Average Light Spirit	Light Spirit	37.5	35	Large Single	1.3	8	10.4
Smirnoff Red Vodka; Gordon's Gin; Bacardi; Glen Vodka; London Gin Distiller's Vodka; Vladivar Classic Vodka; Chekov & Other Average Light Spirit	Light Spirit	37.5	50	Small Double	1.9	8	15.2
Smirnoff Red Vodka; Gordon's Gin; Bacardi; Glen Vodka; London Gin Distiller's Vodka; Vladivar Classic Vodka; Chekov & Other Average Light Spirit	Light Spirit	37.5	70	Large Double	2.6	8	20.8
Smirnoff Red Vodka; Gordon's Gin; Bacardi; Glen Vodka; London Gin Distiller's Vodka; Vladivar Classic Vodka; Chekov & Other Average Light Spirit	Light Spirit	37.5	1000	1 Litre Bottle	37.5	8	300
Smirnoff Red Vodka; Gordon's Gin; Bacardi; Glen Vodka; London Gin Distiller's Vodka; Vladivar Classic Vodka; Chekov & Other Average Light Spirit	Light Spirit	37.5	700	Bottle	26.3	8	210.4
Smirnoff Red Vodka; Gordon's Gin; Bacardi; Glen Vodka; London Gin Distiller's Vodka; Vladivar Classic Vodka; Chekov & Other Average Light Spirit	Light Spirit	37.5	350	Half Bottle	13.1	8	104.8
Captain Morgan; Bombay Sapphire Gin; Captain Morgans Spiced & Absolut Vodka	Light Spirit	40	25	Small Single	1	8	8

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Captain Morgan; Bombay Sapphire Gin; Captain Morgans Spiced & Absolut Vodka	Light Spirit	40	35	Large Single	1.4	8	11.2
Captain Morgan; Bombay Sapphire Gin; Captain Morgans Spiced & Absolut Vodka	Light Spirit	40	50	Small Double	2	8	16
Captain Morgan; Bombay Sapphire Gin; Captain Morgans Spiced & Absolut Vodka	Light Spirit	40	70	Large Double	2.8	8	22.4
Captain Morgan; Bombay Sapphire Gin; Captain Morgans Spiced & Absolut Vodka	Light Spirit	40	1000	1 Litre Bottle	40	8	320
Captain Morgan; Bombay Sapphire Gin; Captain Morgans Spiced & Absolut Vodka	Light Spirit	40	700	Bottle	28	8	224
Captain Morgan; Bombay Sapphire Gin; Captain Morgans Spiced & Absolut Vodka	Light Spirit	40	350	Half Bottle	14	8	112

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Bells; Famous Grouse; Teachers; William Grants; Jack Daniels; Martell VS; Courvoisier; Three Barrels; Whyte & Mackay; Glenfiddich SR & Other Average Dark Spirit	Dark Spirit	40	25	Small Single	1	8	8
Bells; Famous Grouse; Teachers; William Grants; Jack Daniels; Martell VS; Courvoisier; Three Barrels; Whyte & Mackay; Glenfiddich SR & Other Average Dark Spirit	Dark Spirit	40	35	Large Single	1.4	8	11
Bells; Famous Grouse; Teachers; William Grants; Jack Daniels; Martell VS; Courvoisier; Three Barrels; Whyte & Mackay; Glenfiddich SR & Other Average Dark Spirit	Dark Spirit	40	50	Small Double	2	8	16
Bells; Famous Grouse; Teachers; William Grants; Jack Daniels; Martell VS; Courvoisier; Three Barrels; Whyte & Mackay; Glenfiddich SR & Other Average Dark Spirit	Dark Spirit	40	70	Large Double	2.8	8	22
Bells; Famous Grouse; Teachers; William Grants; Jack Daniels; Martell VS; Courvoisier; Three Barrels; Whyte & Mackay; Glenfiddich SR & Other Average Dark Spirit	Dark Spirit	40	1000	1 Litre Bottle	40	8	320
Bells; Famous Grouse; Teachers; William Grants; Jack Daniels; Martell VS; Courvoisier; Three Barrels; Whyte & Mackay; Glenfiddich SR & Other Average Dark Spirit	Dark Spirit	40	700	Bottle	28	8	224
Bells; Famous Grouse; Teachers; William Grants; Jack Daniels; Martell VS; Courvoisier; Three Barrels; Whyte & Mackay; Glenfiddich SR & Other Average Dark Spirit	Dark Spirit	40	350	Half Bottle	14	8	112

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Other Average Fortified Wine	Fortified Wine	18	50	Glass	0.9	8	7.2
Other Average Fortified Wine	Fortified Wine	18	750	Bottle	14	8	108
Harveys Bristol Cream & Croft	Fortified Wine	17.5	50	Glass	0.9	8	7.2
Harveys Bristol Cream & Croft	Fortified Wine	17.5	750	Bottle	13	8	105
Martinin Rosso; Martinin Rosso; Martini Extra Dry & QC	Fortified Wine	15	50	Glass	0.8	8	6.4
Martinin Rosso; Martinin Rosso; Martini Extra Dry & QC	Fortified Wine	15	750	Bottle	11	8	90
Cockburns Reserve; Grahams LBV; Dows LBV & Taylors LBV	Fortified Wine	20	50	Glass	1	8	8
Cockburns Reserve; Grahams LBV; Dows LBV & Taylors LBV	Fortified Wine	20	750	Bottle	15	8	120
Buckfast	Tonic Wine	15	750	Bottle	11	8	90

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Moet; Veuve Cliquot; Jacobs Creek Sparkling; Mumm; Bollingner; Piper Heidsieck; Heidieck Monopole & Other Average Champagne & Sparkling Wine	Champagne & Sparkling Wine	12	125	Small Glass	1.5	8	12
Moet; Veuve Cliquot; Jacobs Creek Sparkling; Mumm; Bollingner; Piper Heidsieck; Heidieck Monopole & Other Average Champagne & Sparkling Wine	Champagne & Sparkling Wine	12	175	Standard Glass	2.1	8	16.8
Moet; Veuve Cliquot; Jacobs Creek Sparkling; Mumm; Bollingner; Piper Heidsieck; Heidieck Monopole & Other Average Champagne & Sparkling Wine	Champagne & Sparkling Wine	12	250	Large Glass	3	8	24
Moet; Veuve Cliquot; Jacobs Creek Sparkling; Mumm; Bollingner; Piper Heidsieck; Heidieck Monopole & Other Average Champagne & Sparkling Wine	Champagne & Sparkling Wine	12	375	Half Bottle	4.5	8	36
Moet; Veuve Cliquot; Jacobs Creek Sparkling; Mumm; Bollingner; Piper Heidsieck; Heidieck Monopole & Other Average Champagne & Sparkling Wine	Champagne & Sparkling Wine	12	750	Bottle	9	8	72
Lanson	Champagne & Sparkling Wine	12.5	125	Small Glass	1.6	8	12.8
Lanson	Champagne & Sparkling Wine	12.5	175	Standard Glass	2.2	8	17.6
Lanson	Champagne & Sparkling Wine	12.5	250	Large Glass	3.1	8	24.8
Lanson	Champagne & Sparkling Wine	12.5	375	Half Bottle	4.7	8	37.6
Lanson	Champagne & Sparkling Wine	12.5	750	Bottle	9.4	8	75.2

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Freixnet	Champagne & Sparkling Wine	11.5	125	Small Glass	1.4	8	11.2
Freixnet	Champagne & Sparkling Wine	11.5	175	Standard Glass	2	8	16
Freixnet	Champagne & Sparkling Wine	11.5	250	Large Glass	2.9	8	23.2
Freixnet	Champagne & Sparkling Wine	11.5	375	Half Bottle	4.3	8	34.4
Freixnet	Champagne & Sparkling Wine	11.5	750	Bottle	8.6	8	68.8

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Nottage Hill Chardonnay; Trulli (Pinot Grigio); Banrock Station Colombard Chardonnay; Blossom Hill (Californian White) & Other Average White Wine	White Wine	12	125	Small Glass	1.5	8	12
Nottage Hill Chardonnay; Trulli (Pinot Grigio); Banrock Station Colombard Chardonnay; Blossom Hill (Californian White) & Other Average White Wine	White Wine	12	175	Standard Glass	2.1	8	16.8
Nottage Hill Chardonnay; Trulli (Pinot Grigio); Banrock Station Colombard Chardonnay; Blossom Hill (Californian White) & Other Average White Wine	White Wine	12	250	Large Glass	3	8	24
Nottage Hill Chardonnay; Trulli (Pinot Grigio); Banrock Station Colombard Chardonnay; Blossom Hill (Californian White) & Other Average White Wine	White Wine	12	375	Half Bottle	4.5	8	36
Nottage Hill Chardonnay; Trulli (Pinot Grigio); Banrock Station Colombard Chardonnay; Blossom Hill (Californian White) & Other Average White Wine	White Wine	12	750	Bottle	9	8	72
Wolf Blass Chardonnay	White Wine	13.5	125	Small Glass	1.7	8	13.6
Wolf Blass Chardonnay	White Wine	13.5	175	Standard Glass	2.4	8	19.2
Wolf Blass Chardonnay	White Wine	13.5	250	Large Glass	3.4	8	27.2
Wolf Blass Chardonnay	White Wine	13.5	375	Half Bottle	5.1	8	40.8
Wolf Blass Chardonnay	White Wine	13.5	750	Bottle	10.1	8	80.8
Jacobs Creek Chardonnay & Average 13% White Wine	White Wine	13	125	Small Glass	1.6	8	12.8
Jacobs Creek Chardonnay & Average 13% White Wine	White Wine	13	175	Standard Glass	2.3	8	18.4

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Jacobs Creek Chardonnay & Average 13% White Wine	White Wine	13	250	Large Glass	3.3	8	26.4
Jacobs Creek Chardonnay & Average 13% White Wine	White Wine	13	375	Half Bottle	4.9	8	39.2
Jacobs Creek Chardonnay & Average 13% White Wine	White Wine	13	750	Bottle	9.8	8	78.4
Jacobs Creek Semillion Chardonnay	White Wine	11.5	125	Small Glass	1.4	8	11.2
Jacobs Creek Semillion Chardonnay	White Wine	11.5	175	Standard Glass	2	8	16
Jacobs Creek Semillion Chardonnay	White Wine	11.5	250	Large Glass	2.9	8	23.2
Jacobs Creek Semillion Chardonnay	White Wine	11.5	375	Half Bottle	4.3	8	34.4
Jacobs Creek Semillion Chardonnay	White Wine	11.5	750	Bottle	8.6	8	68.8
Montana (Sauvingon Blanc)	White Wine	12.9	125	Small Glass	1.6	8	12.8
Montana (Sauvingon Blanc)	White Wine	12.9	175	Standard Glass	2.3	8	18.4
Montana (Sauvingon Blanc)	White Wine	12.9	250	Large Glass	3.2	8	25.6
Montana (Sauvingon Blanc)	White Wine	12.9	375	Half Bottle	4.8	8	38.4
Montana (Sauvingon Blanc)	White Wine	12.9	750	Bottle	9.7	8	77.6
Average 9% White Wine	White Wine	9	125	Small Glass	1.1	8	8.8
Average 9% White Wine	White Wine	9	175	Standard Glass	1.6	8	12.8

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Average 9% White Wine	White Wine	9	250	Large Glass	2.3	8	18.4
Average 9% White Wine	White Wine	9	375	Half Bottle	3.4	8	27.2
Average 9% White Wine	White Wine	9	750	Bottle	6.8	8	54.4
Average 10% White Wine	White Wine	10	125	Small Glass	1.3	8	10.4
Average 10% White Wine	White Wine	10	175	Standard Glass	1.8	8	14.4
Average 10% White Wine	White Wine	10	250	Large Glass	2.5	8	20
Average 10% White Wine	White Wine	10	375	Half Bottle	3.8	8	30.4
Average 10% White Wine	White Wine	10	750	Bottle	7.5	8	60
Average 11% White Wine	White Wine	11	125	Small Glass	1.4	8	11.2
Average 11% White Wine	White Wine	11	175	Standard Glass	1.9	8	15.2
Average 11% White Wine	White Wine	11	250	Large Glass	2.8	8	22.4
Average 11% White Wine	White Wine	11	375	Half Bottle	4.1	8	32.8
Average 11% White Wine	White Wine	11	750	Bottle	8.3	8	66.4
Average 14% White Wine	White Wine	14	125	Small Glass	1.8	8	14.4
Average 14% White Wine	White Wine	14	175	Standard Glass	2.5	8	20

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Average 14% White Wine	White Wine	14	250	Large Glass	3.5	8	28
Average 14% White Wine	White Wine	14	375	Half Bottle	5.3	8	42.4
Average 14% White Wine	White Wine	14	750	Bottle	9	8	72

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Ernest & Julio Gallo Merlot	Red Wine	12.5	125	Small Glass	1.6	8	13
Ernest & Julio Gallo Merlot	Red Wine	12.5	175	Standard Glass	2.2	8	18
Ernest & Julio Gallo Merlot	Red Wine	12.5	250	Large Glass	3.1	8	25
Ernest & Julio Gallo Merlot	Red Wine	12.5	375	Half Bottle	4.7	8	38
Ernest & Julio Gallo Merlot	Red Wine	12.5	750	Bottle	9.4	8	75
Campo Viejo (Rioja); Jacobs Creek (Shiraz Cabernet) & Wolf Blass (Shiraz Cabernet)	Red Wine	13	125	Small Glass	1.6	8	13
Campo Viejo (Rioja); Jacobs Creek (Shiraz Cabernet) & Wolf Blass (Shiraz Cabernet)	Red Wine	13	175	Standard Glass	2.3	8	18
Campo Viejo (Rioja); Jacobs Creek (Shiraz Cabernet) & Wolf Blass (Shiraz Cabernet)	Red Wine	13	250	Large Glass	3.3	8	26
Campo Viejo (Rioja); Jacobs Creek (Shiraz Cabernet) & Wolf Blass (Shiraz Cabernet)	Red Wine	13	375	Half Bottle	4.9	8	39
Campo Viejo (Rioja); Jacobs Creek (Shiraz Cabernet) & Wolf Blass (Shiraz Cabernet)	Red Wine	13	750	Bottle	9.8	8	78
9% Red/Rose Wine	Red/Rose Wine	9	125	Small Glass	1.1	8	8.8
9% Red/Rose Wine	Red/Rose Wine	9	175	Standard Glass	1.6	8	13
9% Red/Rose Wine	Red/Rose Wine	9	250	Large Glass	2.3	8	18
9% Red/Rose Wine	Red/Rose Wine	9	375	Half Bottle	3.4	8	27
9% Red/Rose Wine	Red/Rose Wine	9	750	Bottle	6.8	8	54
10% Red/Rose Wine	Red/Rose Wine	10	125	Small Glass	1.3	8	10
10% Red/Rose Wine	Red/Rose Wine	10	175	Standard Glass	1.8	8	14
10% Red/Rose Wine	Red/Rose Wine	10	250	Large Glass	2.5	8	20
10% Red/Rose Wine	Red/Rose Wine	10	375	Half Bottle	3.8	8	30
10% Red/Rose Wine	Red/Rose Wine	10	750	Bottle	7.5	8	60

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
11% Red/Rose Wine	Red/Rose Wine	11	125	Small Glass	1.4	8	11
11% Red/Rose Wine	Red/Rose Wine	11	175	Standard Glass	1.9	8	15
11% Red/Rose Wine	Red/Rose Wine	11	250	Large Glass	2.8	8	22
11% Red/Rose Wine	Red/Rose Wine	11	375	Half Bottle	4.1	8	33
11% Red/Rose Wine	Red/Rose Wine	11	750	Bottle	8.3	8	66
12% Red/Rose Wine	Red/Rose Wine	12	125	Small Glass	1.5	8	12
12% Red/Rose Wine	Red/Rose Wine	12	175	Standard Glass	2.1	8	17
12% Red/Rose Wine	Red/Rose Wine	12	250	Large Glass	3	8	24
12% Red/Rose Wine	Red/Rose Wine	12	375	Half Bottle	4.5	8	36
12% Red/Rose Wine	Red/Rose Wine	12	750	Bottle	9	8	72
14% Red/Rose Wine	Red/Rose Wine	14	125	Small Glass	1.8	8	14
14% Red/Rose Wine	Red/Rose Wine	14	175	Standard Glass	2.5	8	20
14% Red/Rose Wine	Red/Rose Wine	14	250	Large Glass	3.5	8	28
14% Red/Rose Wine	Red/Rose Wine	14	375	Half Bottle	5.3	8	42
14% Red/Rose Wine	Red/Rose Wine	14	750	Bottle	10.5	8	84

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Strongbow	Cider/Perry	5.3	330	Bottle	1.7	8	13.6
Strongbow	Cider/Perry	5.3	440	Can	2.3	8	18.4
Strongbow	Cider/Perry	5.3	284	Half Pint	1.5	8	12.0
Strongbow	Cider/Perry	5.3	568	Pint	3.0	8	24.0
Lambrini; White Lightening; Diamond White & Frosty Jack	Cider/Perry	7.5	330	Bottle	2.5	8	20.0
Lambrini; White Lightening; Diamond White & Frosty Jack	Cider/Perry	7.5	440	Can	3.3	8	26.4
Lambrini; White Lightening; Diamond White & Frosty Jack	Cider/Perry	7.5	284	Half Pint	2.1	8	16.8
Lambrini; White Lightening; Diamond White & Frosty Jack	Cider/Perry	7.5	568	Pint	3.4	8	27.2
Scrumpy Jack & Blackthorn	Cider/Perry	6	330	Bottle	2.0	8	16.0
Scrumpy Jack & Blackthorn	Cider/Perry	6	440	Can	2.6	8	20.8
Scrumpy Jack & Blackthorn	Cider/Perry	6	284	Half Pint	1.7	8	13.6
Scrumpy Jack & Blackthorn	Cider/Perry	6	568	Pint	3.4	8	27.2
Gaymer Olde English and Jacques	Cider/Perry	5.5	330	Bottle	1.8	8	14.4
Gaymer Olde English and Jacques	Cider/Perry	5.5	440	Can	2.4	8	19.2
Gaymer Olde English and Jacques	Cider/Perry	5.5	284	Half Pint	1.6	8	12.8
Gaymer Olde English and Jacques	Cider/Perry	5.5	568	Pint	3.1	8	24.8
Woodpecker	Cider/Perry	3.5	330	Bottle	1.2	8	9.6
Woodpecker	Cider/Perry	3.5	440	Can	1.5	8	12.0
Woodpecker	Cider/Perry	3.5	284	Half Pint	1.0	8	8.0
Woodpecker	Cider/Perry	3.5	568	Pint	2.0	8	16.0
St Heliers Pear Cider and Other Average Cider/Perry	Cider/Perry	5	330	Bottle	1.7	8	13.6
St Heliers Pear Cider and Other Average Cider/Perry	Cider/Perry	5	440	Can	2.2	8	17.6

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
St Heliers Pear Cider and Other Average Cider/Perry	Cider/Perry	5	284	Half Pint	1.4	8	11.2
St Heliers Pear Cider and Other Average Cider/Perry	Cider/Perry	5	568	Pint	2.8	8	22.4
Magners, Bulmers, Kopparberg Pear	Cider/Perry	4.5	330	Bottle	1.5	8	12.0
Magners, Bulmers, Kopparberg Pear	Cider/Perry	4.5	440	Can	2.0	8	15.8
Magners, Bulmers, Kopparberg Pear	Cider/Perry	4.5	284	Half Pint	1.3	8	10.4
Magners, Bulmers, Kopparberg Pear	Cider/Perry	4.5	568	Pint	2.6	8	20.5
Thatcher's Katy Cider	Cider/Perry	7.4	330	Bottle	2.4	8	19.2
Thatcher's Katy Cider	Cider/Perry	7.4	440	Can	3.3	8	26.0
Thatcher's Katy Cider	Cider/Perry	7.4	284	Half Pint	2.1	8	16.8
Thatcher's Katy Cider	Cider/Perry	7.4	568	Pint	4.2	8	33.6

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Carlsberg Special Brew	Lager	9	330	Bottle	3.0	8	24
Carlsberg Special Brew	Lager	9	440	Can	4.0	8	32
Carlsberg Special Brew	Lager	9	284	Half Pint	2.6	8	20.8
Carlsberg Special Brew	Lager	9	568	Pint	5.1	8	40.8
Stella	Lager	5.2	330	Bottle	1.7	8	13.6
Stella	Lager	5.2	440	Can	2.3	8	18.4
Stella	Lager	5.2	284	Half Pint	1.5	8	12
Stella	Lager	5.2	568	Pint	3.0	8	24
Carling	Lager	4.1	330	Bottle	1.4	8	11.2
Carling	Lager	4.1	440	Can	1.8	8	14.4
Carling	Lager	4.1	284	Half Pint	1.2	8	9.6
Carling	Lager	4.1	568	Pint	2.3	8	18.4
Fosters, Carlsberg and Tennants	Lager	4	330	Bottle	1.3	8	10.4
Fosters, Carlsberg and Tennants	Lager	4	440	Can	1.8	8	14.4
Fosters, Carlsberg and Tennants	Lager	4	284	Half Pint	1.1	8	8.8
Fosters, Carlsberg and Tennants	Lager	4	568	Pint	2.3	8	18.4
Budweiser; Kronenbourg; Grolsch; Becks; Carlsberg Export; Heineken, Asahi, Cobra and Straropramen & Other Average Lager	Lager	5	330	Bottle	1.7	8	13.6
Budweiser; Kronenbourg; Grolsch; Becks; Carlsberg Export; Heineken, Asahi, Cobra and Straropramen & Other Average Lager	Lager	5	440	Can	2.2	8	17.6
Budweiser; Kronenbourg; Grolsch; Becks; Carlsberg Export; Heineken, Asahi, Cobra and Straropramen & Other Average Lager	Lager	5	284	Half Pint	1.4	8	11.2
Budweiser; Kronenbourg; Grolsch; Becks; Carlsberg Export; Heineken, Asahi, Cobra and Straropramen & Other Average Lager	Lager	5	568	Pint	2.8	8	22.4
Corona	Lager	4.6	330	Bottle	1.5	8	12
Corona	Lager	4.6	440	Can	2.0	8	16
Corona	Lager	4.6	284	Half Pint	1.3	8	10.4
Corona	Lager	4.6	568	Pint	2.6	8	20.8

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Pilsner	Lager	4.4	330	Bottle	1.5	8	11.6
Pilsner	Lager	4.4	440	Can	1.9	8	15.2
Pilsner	Lager	4.4	284	Half Pint	1.2	8	9.6
Pilsner	Lager	4.4	568	Pint	2.5	8	20
Peroni	Lager	5.3	330	Bottle	1.7	8	13.6
Peroni	Lager	5.3	440	Can	2.3	8	18.4
Peroni	Lager	5.3	284	Half Pint	1.5	8	12
Peroni	Lager	5.3	568	Pint	3.0	8	24
Baltika	Lager	5.1	330	Bottle	1.7	8	13.6
Baltika	Lager	5.1	440	Can	2.2	8	17.6
Baltika	Lager	5.1	284	Half Pint	1.4	8	11.2
Baltika	Lager	5.1	568	Pint	2.9	8	23.2
Miller	Lager	4.7	330	Bottle	1.6	8	12.4
Miller	Lager	4.7	440	Can	2.1	8	16.8
Miller	Lager	4.7	284	Half Pint	1.3	8	10.4
Miller	Lager	4.7	568	Pint	2.7	8	21.6

Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
John Smiths; Stones & Other Average Beer/Ale	Beer/Ale	4	330	Bottle	1.3	8	10.4
John Smiths; Stones & Other Average Beer/Ale	Beer/Ale	4	440	Can	1.8	8	14.4
John Smiths; Stones & Other Average Beer/Ale	Beer/Ale	4	284	Half Pint	1.1	8	8.8
John Smiths; Stones & Other Average Beer/Ale	Beer/Ale	4	568	Pint	2.3	8	18.4
Guinness & Caffreys	Beer/Ale	4.2	330	Bottle	1.4	8	11.2
Guinness & Caffreys	Beer/Ale	4.2	440	Can	1.8	8	14.4
Guinness & Caffreys	Beer/Ale	4.2	284	Half Pint	1.2	8	9.6
Guinness & Caffreys	Beer/Ale	4.2	568	Pint	2.4	8	19.2
Boddingtons & Tetleys	Beer/Ale	3.8	330	Bottle	1.3	8	10.4
Boddingtons & Tetleys	Beer/Ale	3.8	440	Can	1.7	8	13.6
Boddingtons & Tetleys	Beer/Ale	3.8	284	Half Pint	1.1	8	8.8
Boddingtons & Tetleys	Beer/Ale	3.8	568	Pint	2.2	8	17.6
McEwans Export & Old Speckled Hen	Beer/Ale	4.5	330	Bottle	1.5	8	12.0
McEwans Export & Old Speckled Hen	Beer/Ale	4.5	440	Can	2.0	8	16.0
McEwans Export & Old Speckled Hen	Beer/Ale	4.5	284	Half Pint	1.3	8	10.4
McEwans Export & Old Speckled Hen	Beer/Ale	4.5	568	Pint	2.7	8	21.6
Newcastle Brown	Beer/Ale	4.7	330	Bottle	1.6	8	12.8
Newcastle Brown	Beer/Ale	4.7	440	Can	2.1	8	16.8
Newcastle Brown	Beer/Ale	4.7	284	Half Pint	1.3	8	10.4
Newcastle Brown	Beer/Ale	4.7	568	Pint	2.7	8	21.6
Worthington	Beer/Ale	3.6	330	Bottle	1.2	8	9.6
Worthington	Beer/Ale	3.6	440	Can	1.6	8	12.8
Worthington	Beer/Ale	3.6	284	Half Pint	1.0	8	8.0
Worthington	Beer/Ale	3.6	568	Pint	2.0	8	16.0
Belhaven Best and Tennents Special	Beer/Ale	3.5	330	Bottle	1.2	8	9.2

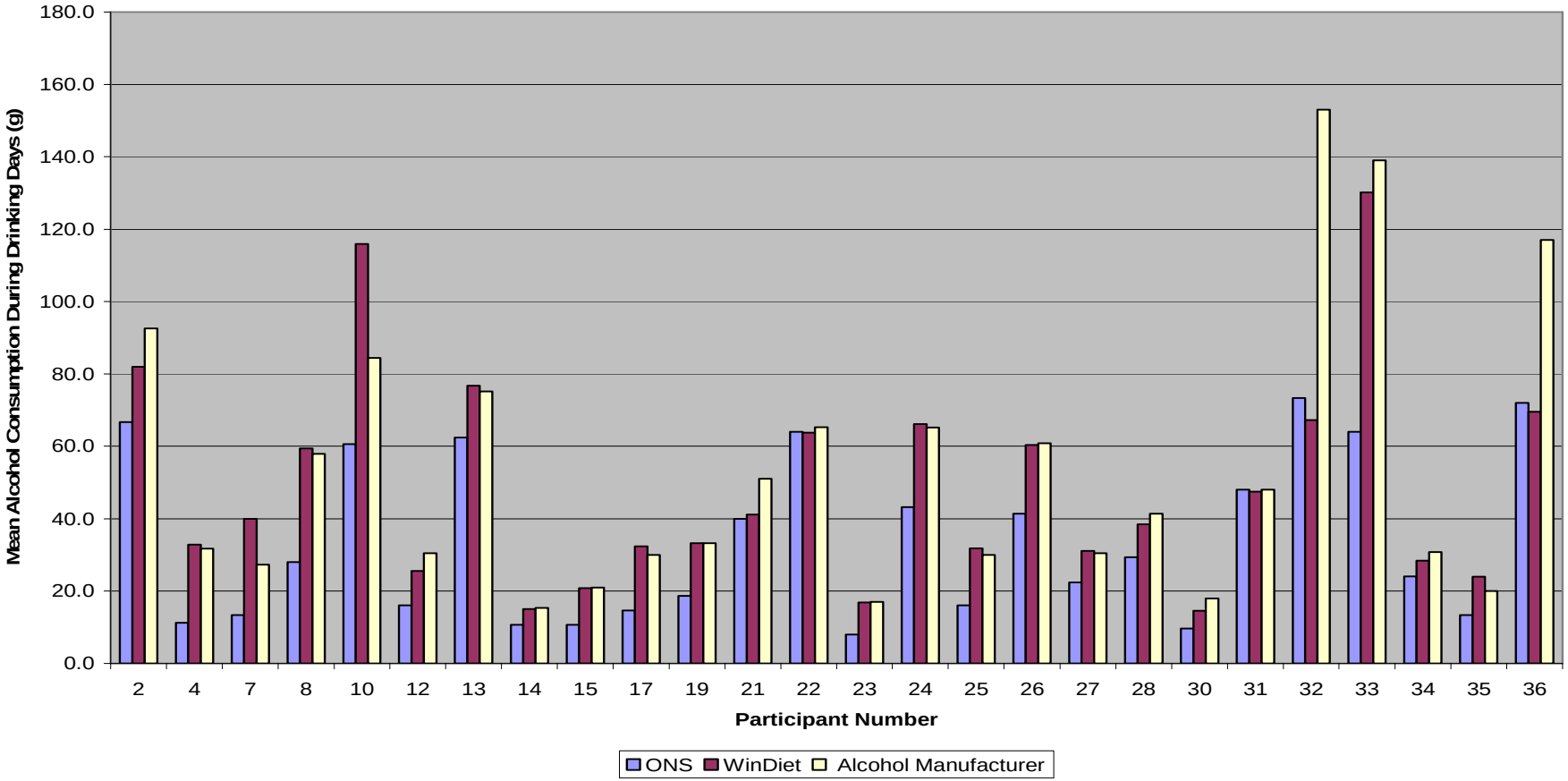
Name of Drink	Type of Drink	% ABV	Volume (ml)	Measurement	No of Units	UK Standard Alcohol Content (g)/Unit	Alcohol content (g)/Drink
Belhaven Best and Tennents Special	Beer/Ale	3.5	440	Can	1.5	8	12.0
Belhaven Best and Tennents Special	Beer/Ale	3.5	284	Half Pint	1.0	8	7.9
Belhaven Best and Tennents Special	Beer/Ale	3.5	568	Pint	2.0	8	15.8
Caledonian -80/	Beer/Ale	4.1	330	Bottle	1.4	8	10.8
Caledonian -80/	Beer/Ale	4.1	440	Can	1.8	8	14.4
Caledonian -80/	Beer/Ale	4.1	284	Half Pint	1.2	8	9.6
Caledonian -80/	Beer/Ale	4.1	568	Pint	2.3	8	18.4

APPENDIX 3

A. Table of Participants Mean Alcohol Consumption (g) During Drinking Days (N=26)

		Mean Alcohol Consumption During Drinking Days (g) by Analysis Method		
Participant No.	**No. of Drinking Days**	**ONS**	**WinDiets**	**Alcohol Manufacturer**
2	6	66.7	81.9	92.5
4	5	11.2	32.8	31.7
7	3	13.3	40.0	27.3
8	4	28.0	59.4	57.9
10	7	60.6	115.9	84.4
12	5	16.0	25.6	30.5
13	5	62.4	76.7	75.1
14	3	10.7	15.0	15.4
15	6	10.7	20.8	20.9
17	6	14.7	32.4	30.0
19	6	18.7	33.2	33.3
21	4	40.0	41.1	51.0
22	3	64.0	63.7	65.3
23	1	8.0	16.8	17.0
24	5	43.2	66.1	65.2
25	2	16.0	31.8	30.0
26	6	41.3	60.4	60.8
27	5	22.4	31.1	30.5
28	3	29.3	38.4	41.3
30	5	9.6	14.6	17.9
31	1	48.0	47.5	48.0
32	6	73.3	67.3	153.0
33	1	64.0	130.2	139.0
34	2	24.0	28.4	30.8
35	3	13.3	24.0	20.0
36	1	72.0	69.5	117.0

B. Bar graph of HI study drinkers (N=26) mean alcohol consumption during drinking days calculated by three differing procedures.



APPENDIX 4

A. Published Abstract: Alcohol Consumption And Cardiovascular Disease Risk – Depends How You Measure It?

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Introduction

The abuse of alcohol is a major public health concern within the U.K. and has been linked to a wide range of health conditions including increased risk of cardiovascular disease (CVD). The U.K. Department of Health recommends limiting daily alcohol consumption to 24-32g (male) and 16-24g (female). Any attempt to provide a reliable and credible estimate of the disease risk associated with intake of alcohol is critically dependent on the accuracy of the method employed to record consumption. The aim of the present work was to compare the accuracy of three analytical methods of assessing alcohol consumption in a population sample who recorded their drinking prospectively in a 7 day diary.

Methods

Data (derived from the first author's PhD study) from 26 participants (18 female and 8 male), who consumed alcohol were analysed. Mean alcohol consumption during drinking days was calculated in grams using the following three analytical methods: alcohol drink unit measurements from the Office of National Statistics (ONS) questionnaire; WinDiets dietary software (WD) and Alcohol Manufacturer's (AM) information. Female participants data were further categorised according to the following drinking guidelines: "sensible" (<24g); "exceeding daily limits" (24-47g) and "binge" (>48g) at one session using AM. Female participants daily alcohol consumption was categorised using WD and ONS and compared to AM.

Results

The total sample had a mean alcohol consumption during drinking days of: AM=53.3g (SD 37.5); WD=48.6g (SD 29.6); ONS=33.9g (SD 22.8). The consideration of %ABV and drink sizes will produce the most accurate estimate of alcohol intake; the AM method. WD underestimates average consumption by 8.8%, ONS by 36.4%. Student T-test was significant (p=<0.001) for ONS compared to AM, but was not significant for WD comparison (p=0.258). Female participant's

daily alcohol consumption was categorised according to UK daily limits. WD and ONS miscategorised 11% and 55% of participants, respectively.

Discussion

The present findings indicate that in studies dependent on participant recall of alcohol intake, a failure to account for individual drink sizes and %ABV, may seriously underestimate intake and the proportion of drinkers exceeding safe guidelines. Investigations which explore the relationship between alcohol intake and CVD disease risk will benefit from measures which improve the accuracy of the quantification of alcohol intake.

B. Poster Presented at the 32nd Annual Scientific Meeting of the Research Society on Alcoholism, June 2009, San Diego, California, USA.